Populations under stress

Analysis on the interface between ecology and evolutionary genetics in nematodes

Agnieszka Doroszuk

Promotor:
Prof. dr. ir. J. Bakker
Hoogleraar in de Nematologie
i.h.b. fysiologie en moleculaire ecologie van nematoden
Wageningen Universiteit

Co-promotor:
Dr. ir. J.E. Kammenga
Universitair hoofddocent bij het Laboratorium voor Nematologie
Wageningen Universiteit

Promotiecommissie:
Prof. dr. L.W. Beukeboom, Rijksuniversiteit, Groningen
Prof. dr. R.F. Hoekstra, Wageningen Universiteit
Dr. P.C. Phillips, University of Oregon, USA
Prof. dr. N.M. van Straalen, Vrije Universiteit, Amsterdam

Dit onderzoek is uitgevoerd binnen de onderzoekschool 'Production Ecology and Resource Conservation'.

Populations under stress

Analysis on the interface between ecology and evolutionary genetics in nematodes

Agnieszka Doroszuk

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. M. J. Kropff
in het openbaar te verdedigen
op dinsdag 1 mei 2007
des middags om 13.30u in de Aula.

Doroszuk, A. (2007).

Populations under stress. Analysis on the interface between ecology and evolutionary genetics in nematodes.

PhD Thesis Wageningen University, Wageningen, The Netherlands, with references, with summaries in English and Dutch.

ISBN: 90-8504-603-3

To André
… i dla rodzinki

Contents

Chapter 1

General introduction

Agnieszka Doroszuk

Effects of adverse environmental conditions are complex, multi-level and might concern short or long time-spans. The effects of stress on lower biological organisation levels such as gene expression and metabolic pattern often result in detrimental effects on individual-level. The changes in individual life-history characteristics such as survival, reproduction or maturation can be linked to the consequences on population-level and higher levels of ecological organisation. Stress is also a strong selective pressure and, therefore, may have a significant impact on evolutionary processes. Searching for the links among the levels of biological organisation and integration of the stress-related knowledge across disciplines is important for understanding the complexity of stress responses and their mechanisms. It is also critical for the development of new assays allowing extrapolation of the individual exposure effects to higher organisation levels (Forbes & Calow 2002; De Coen & Janssen 2003).

Stress

It may seem that the term 'stress' is one of the best known biological terms, well understood by non-scientific public. In the scientific literature, however, this term is used in different contexts and the definition of stress has been the subject of a vivid debate (Grime 1989; Bijlsma & Loeschcke 2005). The way stress is defined depends mainly on the level of biological organization investigated (molecular level, tissue, organism or population) and on research discipline of the study. In physiological studies, it is often defined as changes in physiology, which occur under unfavourable conditions and induce an alarm response (Larcher 1980), while the definition used in evolutionary studies emphasizes a reduction in fitness under such conditions. The view on stress presented in this thesis is related to the concept of an organism's niche. According to Hutchinson (1957), niche is defined as a set of conditions (such as abiotic factors, food resources, predation etc.) allowing the organism's persistence and reproduction. Within its niche the organism experiences a 'normal' range of ecological variables (Fig. 1). When one (or more) ecological variable departs beyond this tolerated range, the organism experiences stress. Stress is, therefore, a condition which depends both on the stressor and the organism. For example, for many animals, limited access to atmospheric oxygen is a stressful condition, while for many anaerobic organisms atmospheric oxygen is highly toxic.

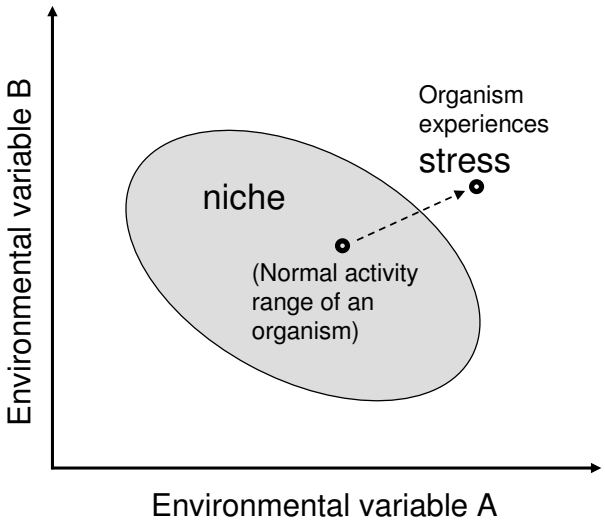


Figure 1: Visualisation of the definition of stress based on the concept of niche.

Human-induced stressors

Environmental stress is not a new phenomenon. In fact, it is as old as life itself and the history of our planet includes the periods when organisms were confronted with adverse conditions on a global scale, periods known as the five major extinctions. Nowadays, the expanding human population and the associated rise in agricultural and industrial activities are the main causes of the rapid loss of biodiversity and the increased pressure on ecosystems (Mooney & Godron 1983; Lawton & May 1995). Protection of threatened populations and ecosystems has become an important public matter. Consequently, big effort has been put into developing methods of risk assessment and their implementation in management strategies. Anthropogenic stressors, including hundred thousands of chemicals (Hansen *et al.* 1999), require efficient toxicity testing protocols that provide straightforward assessment measures (e.g. NOECs, EC50s, LC50s). In response to this need, stress-related research rapidly became dominated by descriptive toxicity studies. During the last years, however, more attention has been directed towards the analyses of stress effects within the frameworks of ecological, evolutionary or genetic research methods (Van Straalen 2003; Bijlsma & Loeschcke 2005). This approach aims to generate testable hypotheses derived from the theoretical knowledge and practical observations. This thesis adopts this view and investigates effects of stress on populations from the perspective of ecological, evolutionary and genetic mechanisms.

A broad view on stress effects

The impact of stress on populations comprises of a variety of interrelated effects. Figure 2 gives an overview of these effects with an emphasis on the aspects relevant for this thesis.

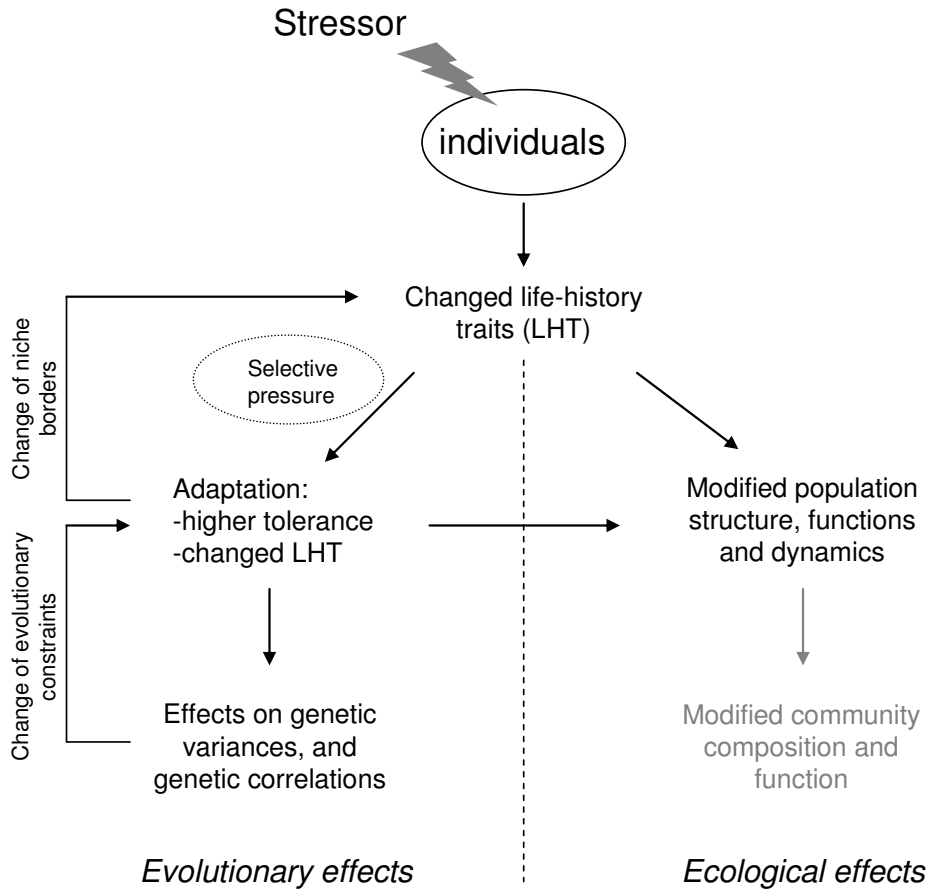


Figure 2: Stress effects on a population and their interrelations.

Presence of a toxic substance or a change in the magnitude of a critical environmental factor (e.g. temperature) creates conditions close or beyond the range tolerated by a population (the population's niche). Under such conditions, organisms respond with the changes on molecular and physiological level, which might affect their survival, reproduction or other individual life-history characteristics. The consequences of life-history alterations are often observed on population parameters like population size, biomass and annual biomass production. Consequently, the effects on higher levels of ecological organisation (community, ecosystem) are expected. Stress is often transient and many physiological and behavioural mechanisms counteract its adverse effects and allow organisms to survive outside their niche for some time. When stress persists, however, organisms might evolve adaptive

mechanisms to reduce its impact. Given a sufficient genetic variation within a population, the selective pressure imposed by a stressor might result in adaptive changes and higher resistance. In this way, the boundaries of the niche are shifted. Apart from the evolution of resistance, strong selection imposed by a stressor might also result in a depletion of genetic variance or in a change of genetic relationships among characters (genetic covariances or correlations). Changes in genetic architecture influence the potential for future adaptation and have profound implications for long-term persistence of populations and species.

Ecological parameters

Protection of populations and ecosystems relies on the understanding of populations' responses to stress. Except for extreme cases when high stress levels result in local extinction, accurate estimation of the impact of stress on a population under field conditions remains a difficult task. One of the common means of evaluating the impact of stressors is population size or related parameters such as population density or total biomass. The downside of these measures is, that only long-term monitoring of the populations of interest together with the populations at reference sites can provide a reliable effect estimation. Population growth rate is a more integrative the measure. Unfortunately, it requires life-history assessments under unrealistic laboratory conditions. Parameters such as annual biomass production and biomass turnover rate relate to functional properties of population and community. The effects on these measures represent the influence of stressors on general population performance and are useful for making inferences about the effects on food webs and ecosystem functions.

Adaptation

All organisms have evolved means to cope with some level of stress. They use mechanisms, such as avoidance behaviour and physiological acclimation (which is often confused with adaptation) that temporally allow them to survive adverse conditions. Acclimation arises after pre-exposure of an organism to detrimental conditions and the acquired resistance can not be inherited.

Fitness reduction is the centre of the evolutionary definition of stress (Bijlsma & Loeschcke 2005). Selective pressure imposed by a stressor is, in principle, strong and might lead to genetic adaptation within few generations. As a result, the range of tolerated environmental variables in the population (e.g. a concentration of the contaminant) is shifted. Such adaptive changes to the man-changed environment

include development of extreme temperature resistance in *Drosophila melanogaster* (Hoffmann *et al.* 2003), insecticide tolerance in several insect species (Woods 1981) and heavy metal tolerance in many plants and some invertebrate species (Antonovics *et al.* 1971; Posthuma & Van Straalen 1993). Although it might seem easy to detect adaptations (for example, by detecting a population that is performing well in a contaminated area), providing evidence that adaptation has taken place is rather a tedious process and requires fulfilling several criteria. Brandon (1991) listed five main components needed to provide a complete adaptation explanation. First, there should be evidence that selection has occurred in the past. Second, it needs to be demonstrated that, based on life-history characteristics, some individuals are better adapted than others. Third, evidence for heritability of the increased tolerance is required. Fourth, the potential influence of gene flow should be known and finally, phylogenetic information is needed to allow distinction between the original and derived state of the character. In case of local adaptation, Kawecki and Ebert (2004) emphasized the importance of reciprocal transplant and common garden experiments to demonstrate improved fitness of local populations in their own habitat. Many reported cases of adaptation lack information to meet all these standards.

While the occurrence of adaptation in natural populations is relatively well documented, more detailed information, such as dynamics of adaptive process or specific conditions promoting or delaying adaptation in natural populations, is scarce. Reproductive mode is one of the most discussed factors influencing dynamics of evolutionary processes (Barraclough *et al.* 2003; Wilke 2004; De Visser & Rozen 2005). In general, asexual populations are expected to have lower adaptive potential than the corresponding sexual populations. Indeed, adaptive advantage of sexual reproduction under novel environmental conditions (thus, under stress) is evoked as one of the main arguments explaining the persistence of sex in nature (West *et al.* 1999; Burt 2000).

Genetic architecture

Genetic variance and covariance

Under stress, population sizes are usually reduced and tolerant genotypes are favoured by selection, which is likely to result in depletion of genetic variance. This situation is expected to have profound effects on subsequent evolutionary responses to new stressors or environmental change. As standing genetic variance determines initial responses to a change in environment, its low levels limit the capability of coping with the new stresses and might eventually lead to population extinction.

The outcome of adaptive processes depends to a large extent on the genetic relations among traits. Because the whole individual is the main unit of selection

(Lewontin 1970) and the traits are genetically interrelated, the evolutionary change in one character is usually accompanied by simultaneous changes in other characters. Genetic correlations (or covariances) among traits determine which combinations of character values are 'available' for selection, defining at the same time the constraints imposed on evolutionary processes. Within the framework of quantitative genetics, the concept of genetic variance-covariance (**G**) matrix summarizes genetic relationships among traits and constitutes a tool to investigate multi-trait evolution. The knowledge of **G** matrix and selection gradients allows prediction of evolutionary outcomes and provides insights into past evolutionary processes (Lande 1979; Lande & Arnold 1983). An important condition of such analyses, however, is the stability of **G** matrices over the time-span of the analysed evolutionary process. After several years of research, there is enough evidence to believe that **G** matrices evolve (Steppan *et al.* 2002; McGuigan 2006). However, there is still little information on the time-span and conditions promoting these changes. Since stressors impose strong selective pressures that can lead to adaptations and affect the magnitude of genetic variance, evolutionary change in response to stress might shape the structure of **G** matrices. While the majority of evolution-oriented studies on stress focuses on the changes in trait means, the impact of stress might reach beyond this level and affect genetic relationships among traits.

Genes that matter

The knowledge about the genes underlying natural genetic variance for stress resistance and stress response is important from both, scientific and practical, perspectives. Although the need for the research in this area was recognized relatively early, there are rather few cases, where the genetic determination of stress resistance is well understood. Most of the successful studies on genetics of stress tolerance led to the conclusion that one or a few major genes are involved (Macnair 1991). The investigated range of stressors (mainly heavy metals and pesticides) and species (mainly plant species) was limited, however, and it is likely that other patterns of genetic architecture of stress response and tolerance exist.

According to Mackay (2001) and Cheverud (2006), a complete description of genetic architecture of any trait requires incorporation of the following aspects: (1) the number of loci that affect the observed variation; (2) their allelic effects; (3) the dominance interactions within a single locus and epistatic interactions with other loci and; (4) pleiotropic effects displayed by the loci and (5) identification of the quantitative trait loci (QTL) alleles at the molecular level. Studying all the listed features of genetic architecture requires application of a specific approach within the framework of quantitative genetics: Quantitative Trait Loci (QTL) analysis. It needs to be mentioned that the term QTL usually refers to a chromosomal segment carrying

not only the gene or genes with an effect on the investigated trait, but also many other genes. Although this analysis originates from an old concept (Sax 1923), its broad application became possible only relatively recently, when high density genetic markers became available and significant advances in quantitative mapping algorithms were made.

QTL mapping

The principle of QTL mapping is relatively simple (Fig. 3). Two parental, inbred strains and molecular polymorphic marker linkage map form the basis of the analysis. The parental strains are crossed and a segregating mapping population is derived. The most commonly used mapping populations include F_2, backcross populations and recombinant inbred lines (RILs). The next phase comprises of the determination of the phenotypes and genotypes (using molecular markers) for all individuals within the mapping population. Various statistical QTL mapping methods can be then applied to look for associations between the phenotype and genetic markers. Finding such association for a marker indicates the presence of a linked QTL.

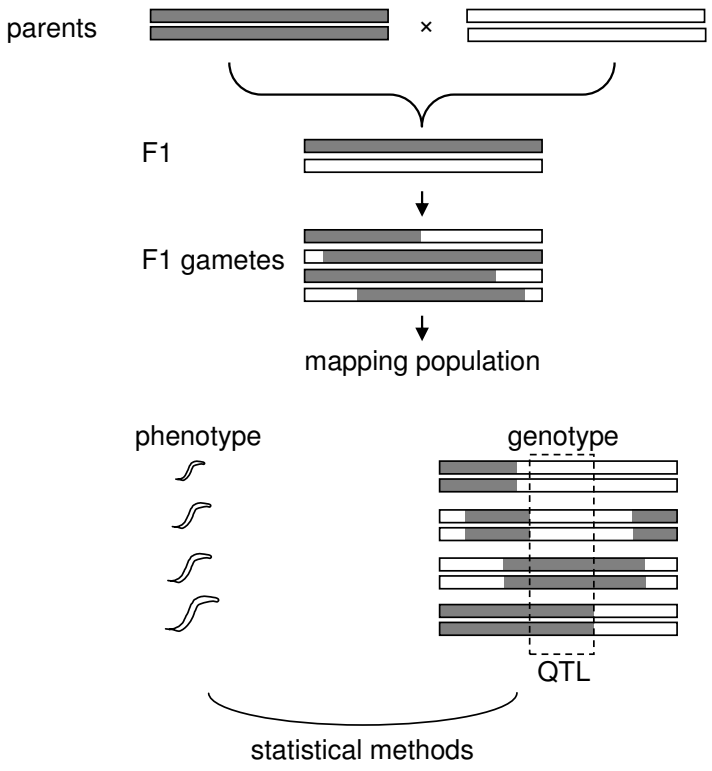


Figure 3: QTL mapping procedure.

Nematodes

Nematodes (roundworms) are one of the most abundant and widely distributed multicellular animal groups on earth (Cobb 1915; Bongers & Schouten 1991). Initially, scientific interest in nematodes reflected the practical need for the protection of agricultural crops from plant parasitic species. Later, however, nematodes became a subject of a broader-scale research, part of which was dedicated to effects of stress on individual, population and community level (e.g. Bongers 1990; Kammenga *et al.* 1998; Jones & Candido 1999; Álvarez *et al.* 2006). In parallel with the growing interest in nematodes in ecological studies, one nematode species, *Caenorhabditis elegans* (Nematoda, Rhabditidae), was beginning its spectacular career in genetics as a model organism.

Acrobeloides nanus

Acrobeloides nanus de Man 1880 (Nematoda, Cephalobidae) is a parthenogenic, free-living, bacterial-feeding nematode, which can be found in soils all over the world (Bird *et al.* 1993; De Goede & Bongers 1998). It can be easily cultured on agar media with bacterial lawns of *Acinetobacter johnsonii* or *Escherichia coli*. Adult individuals are approximately half a millimetre long and under laboratory conditions, at 20 ºC, produce approximately 250 eggs. The eggs are laid within the period of 35-40 days and the average life-span equals 50-60 days. *Acrobeloides nanus* has been a subject of developmental and stress-related studies (e.g. Wiegner & Schierenberg 1998; Laugsch & Schierenberg 2004; Álvarez *et al.* 2006).

Caenorhabditis elegans

Caenorhabditis elegans was introduced to genetic research by Sydney Brenner, who published in 1974 his article on 'the genetics of *Caenorhabditis elegans*' (1974). Nowadays, several hundreds of studies using this species are annually published and the achievements within the *C. elegans* research include the discovery of RNA interference and the advances in the knowledge of programmed cell death and genetics of organ development, which were awarded with the Nobel Prize in 2002 and 2006.

The success of *C. elegans* as a model species can be attributed to features that are attractive from both, economic and research, perspectives. *C. elegans* is easy to rear on agar media seeded with *Escherichia coli*, it has a short generation time (2 days at 20 °C) and can be frozen for storage and subsequently recovered for analysis. It is a multicellular eukaryote with differentiated tissues and, at the same time, a relatively simple organism, which facilitates the research. *C. elegans* reproduces predominantly by self-fertilization. In populations composed of hermaphrodites, males occasionally

occur and the offspring can be produced through mating between the sexes (Hedgecock 1976).

Outline and objectives of the thesis

The thesis is comprised of two main parts. Within the first section I elucidate ecological (**chapter 2**), evolutionary (**chapter 3**) and genetic (**chapter 4**) consequences of long-term stress exposure in wild populations of the nematode *Acrobeloides nanus*. This multi-disciplinary analysis makes use of an experimental field in which the combinations of four levels of pH and copper concentrations were applied approximately 20 years ago in a factorial and replicated manner. **Chapter 2** investigates how secondary production and biomass turnover responded to these stressors. The factorial design of the field experiment allows studying individual- and interactive effects of the stressors. Further, I try to determine whether the changes in these parameters are driven by the changes in biomass, body growth rate or size-frequency distributions of *A. nanus* populations. In **chapter 3,** I test the hypothesis about adaptive changes in life-history traits of *A. nanus*. Specifically, I implement a reciprocal transplant approach and reaction norm experiments to determine whether the populations from two extreme treatments underwent adaptive divergence since the application of the different stress regimes. I discuss the results in the context of the asexual mode of reproduction and deliberate on the adaptive changes in LHT from the perspective of life-history theory. **Chapter 4** is dedicated to study the effect of the applied stressors on genetic variances and genetic covariances among traits (**G** matrix). Similarly to the study presented in **chapter 3**, I focus on the populations from the extreme treatments and provide several tests to determine whether **G** matrices diverged after introducing different stress treatments. Further, I analyze the pattern of the divergence and discuss the role of selection and genetic drift in evolution of **G** matrices.

The second part of the thesis refers to the genetic bases of stress responses and resistance (and other complex traits) investigated within the framework of QTL analysis. In **chapter 5,** I describe a genome-wide library of near-isogenic lines (NILs) constructed from two genetically divergent strains of *Caenorhabditis elegans*, N2 and CB4856. The chapter illustrates the utility of this permanent resource and presents the mapping results of a stress-related response: aggregation behaviour. Subsequently, I discuss the properties of the produced lines and the outcome of the aggregation mapping.

Chapter 6 presents general conclusions of our research and discusses the most important findings and their implications. At the end, the suggestions regarding future research directions are made.

References

Álvarez, O.A., Jager, T., Redondo, E.M. & Kammenga, J.E. (2006) Physiological modes of action of toxic chemicals in the nematode *Acrobeloides nanus*. *Environmental Toxicology and Chemistry*, **25**, 3230-3237.

Antonovics, J., Bradsham, A.D. & Turner, R.G. (1971) Heavy metal tolerance in plants. *Advances in Ecological Research*, **7**, 1–85.

Barraclough, T.G., Birky Jr, C.W. & Burt, A. (2003) Diversification in sexual and asexual organisms. *Evolution*, **57**, 2166.

Bijlsma, R. & Loeschcke, V. (2005) Environmental stress, adaptation and evolution: an overview. *Journal of Evolutionary Biology*, **18**, 744-749.

Bird, A.F., De Ley, P. & Bird, J. (1993) Morphology, oviposition and embryogenesis in an Australian population of *Acrobeloides nanus*. *Journal of Nematology*, **25**, 607-615.

Bongers, T. (1990) The maturity index - an ecological measure of environmental disturbance based on nematode species composition. *Oecologia*, **83**, 14-19.

Bongers, T. & Schouten, T. (1991) Nematodengemeenschappen als potentieel diagnostisch instrument voor chemische verontreinigingen. *Flora en fauna chemisch onder druk* (eds G.P. Hekstra & F.J.M. Van Linden), pp. 175-186. Pudoc, Wageningen.

Brandon, R.N. (1991) *Adaptation and environment* Princeton University Press, Princeton, New Jersey.

Brenner, S. (1974) Genetics of *Caenorhabditis elegans*. *Genetics*, **77**, 71-94.

Burt, A. (2000) Perspective: Sex, recombination, and the efficacy of selection - Was Weismann right? *Evolution*, **54**, 337-351.

Cheverud, J.M. (2006) Genetic architecture of quantitative variation. *Evolutionary genetics, concepts and case studies* (eds C.W. Fox & J.B. Wolf), pp. 288-309. Oxford University Press, Oxford.

Cobb, N.A. (1915) Nematodes and their relationships. *Yearbook of Department of Agriculture for 1914*, pp. 457-490.

De Coen, W.M. & Janssen, C.R. (2003) The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. *Environmental Toxicology and Chemistry*, **22**, 1632-1641.

De Goede, R.G.M. & Bongers, T. (1998) *Nematode communities of northern temperate grassland ecosystems* Focus, Giessen.

De Visser, J.A.G.M. & Rozen, D.E. (2005) Limits to adaptation in asexual populations. *Journal of Evolutionary Biology*, **18**, 779-788.

Forbes, V.E. & Calow, P. (2002) Population growth rate as a basis for ecological risk assessment of toxic chemicals. *Philosophical Transactions: Biological Sciences*, **357**, 1299-1306.

Grime, J.P. (1989) The stress debate - symptom of impending synthesis. *Biological Journal of the Linnean Society*, **37**, 3-17.

Hansen, B.G., Van Haelst, A.G., Van Leeuwen, K. & Van der Zandt, P. (1999) Priority setting for existing chemicals: European Union risk ranking method. *Environmental Toxicology and Chemistry*, **18**, 772-779.

Hedgecock, E.M. (1976) Mating system of *Caenorhabditis elegans* - evolutionary equilibrium between self-fertilization and cross-fertilization in a facultative hermaphrodite. *American Naturalist*, **110**, 1007-1012.

Hoffmann, A.A., Sorensen, J.G. & Loeschcke, V. (2003) Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *Journal of Thermal Biology*, **28**, 175-216.

Hutchinson, G.E. (1957) Population studies - animal ecology and demography - concluding remarks. *Cold Spring Harbor Symposia on Quantitative Biology*, **22**, 415-427.

Jones, D. & Candido, E.P.M. (1999) Feeding is inhibited by sublethal concentrations of toxicants and by heat stress in the nematode *Caenorhabditis elegans*: Relationship to the cellular stress response. *Journal of Experimental Zoology*, **284**, 147-157.

Kammenga, J.E., Arts, M.S.J. & Oude-Breuil, W.J.M. (1998) HSP60 as a potential biomarker of toxic stress in the nematode *Plectus acuminatus*. *Archives of Environmental Contamination and Toxicology*, **34**, 253-258.

Kawecki, T.J. & Ebert, D. (2004) Conceptual issues in local adaptation. *Ecology Letters*, **7**, 1225-1241.

Lande, R. (1979) Quantitative genetic-analysis of multivariate evolution, applied to brain - body size allometry. *Evolution*, **33**, 402-416.

Lande, R. & Arnold, S.J. (1983) The measurement of selection on correlated characters. *Evolution*, **37**, 1210-1226.

Larcher, W. (1980) *Physiological plant ecology* Springer Verlag, New York.

Laugsch, M. & Schierenberg, E. (2004) Differences in maternal supply and early development of closely related nematode species. *International Journal of Developmental Biology*, **48**, 655-662.

Lawton, J.H. & May, R.M. (1995) *Extinction rates* Oxford University Press, Oxford.

Lewontin, R.C. (1970) The units of selection. *Annual Review of Ecology and Systematics*, **1**, 1-18.

Mackay, T.F.C. (2001) Quantitative trait loci in *Drosophila*. *Nature Reviews Genetics*, **2**, 11-20.

Macnair, M.R. (1991) Why the evolution of resistance to anthropogenic toxins normally involves major gene changes: the limits to natural selection. *Genetica*, **V84**, 213-219.

McGuigan, K. (2006) Studying phenotypic evolution using multivariate quantitative genetics. *Molecular Ecology*, **15**, 883-896.

Mooney, H.A. & Godron, M. (1983) *Disturbance and ecosystems - Components of response* Springer-Verlag Berlin, Heidelberg.

Posthuma, L. & Van Straalen, N.M. (1993) Heavy-metal adaptation in terrestrial invertebrates - a review of occurrence, genetics, physiology and ecological consequences. *Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology*, **106**, 11-38.

Sax, K. (1923) The relation between chromosome number, morphological characters and rust resistance in segregates of partially sterile wheat hybrids. *Genetics*, **8**, 301-321.

Steppan, S.J., Phillips, P.C. & Houle, D. (2002) Comparative quantitative genetics: evolution of the G matrix. *Trends in Ecology and Evolution*, **17**, 320-327.

Van Straalen, N. (2003) Ecotoxicology becomes stress ecology. *Environmental Science and Technology*, **37**, 324A-330A.

West, S.A., Lively, C.M. & Read, A.F. (1999) A pluralist approach to sex and recombination. *Journal of Evolutionary Biology*, **12**, 1003-1012.

Wiegner, O. & Schierenberg, E. (1998) Specification of gut cell fate differs significantly between the nematodes *Acrobeloides nanus* and *Caenorhabditis elegans*. *Developmental Biology*, **204**, 3-14.

Wilke, C.O. (2004) The speed of adaptation in large asexual populations. *Genetics*, **167**, 2045-2053.

Woods, R.J. (1981) Insecticide resistance: genes and mechanisms. *Genetic consequences of man made change* (eds J.A. Bishop & L.M. Cook), pp. 53-96. Academic Press, London, UK.

Chapter 2

Response of secondary production and its components to multiple stressors in nematode field populations

Agnieszka Doroszuk
Elske te Brake
David Crespo-Gonzalez
Jan E. Kammenga

2007, *Journal of Applied Ecology, 44(2): 446*

Abstract

The ecological risk assessment of soil contamination is of increasing importance regarding environmental protection and resource conservation. Although multiple anthropogenic stressors are common in human-dominated environments, knowledge of their influence on functional population parameters like secondary production (P) and biomass turnover (P/B) is very limited. Secondary production integrates population characteristics such as biomass, size-frequency distribution and body growth rate and provides a link between population and system ecology. The influence of copper and pH stress on yearly secondary production and biomass turnover of field populations of the nematode *Acrobeloides nanus* was investigated by using a randomized factorial block design. The responses of the components of secondary production were also analysed in order to elucidate the mechanisms underlying the change in secondary production. Secondary production and biomass turnover showed reduced values in soil of low pH. A negative effect of copper on both parameters was observed only when the copper load was combined with low pH, otherwise higher copper concentrations resulted in higher secondary production and biomass turnover. The observed response of production and biomass turnover was mainly driven by the changes in mean relative growth rate (MRGR), a measure of body growth rate estimated in a laboratory soil experiment. The biomass was higher on average in the plots with high copper load while no significant response to pH was found. Our results demonstrate that populations of soil organisms may experience strong synergistic effects of combined stressors (acidification and copper stress) on functional population parameters, while showing no detrimental effects on biomass. Moreover, the effects on secondary production and biomass turnover rate were predominantly driven by effects on body growth rate. We recommend that ecological risk assessment methodologies should include consideration of soil contamination on the basis of conservation of functional properties of ecosystems and their key components.

Introduction

Natural populations are often exposed to combinations of anthropogenic stressors which can have detrimental effects (Yan *et al.* 1996; Breiburg *et al.* 1998). For example, multiple stressors have been recognised as a key factor contributing to the global decline of amphibians and coral reefs (Knowlton 2001; Blaustein & Kiesecker 2002). It is difficult to investigate the effects of multiple stressors on populations under field conditions because of the possible interactive effects between the stressors and indirect effects caused by interactions between populations in natural ecosystems (Caswell 1989; Preston 2002; Sih *et al.* 2004; Salbu *et al.* 2005).

Various population-level measures can be applied to evaluate the impact of a stressor or a combination of stressors on populations. Secondary production (P) is a functional parameter that represents the process in which heterotrophs use part of the energy acquired from the prey for growth and reproduction (Van Straalen 1989), and therefore provides a measure of energy flow through a population. The importance of secondary production follows from the reasoning that the flow of energy through the various groups of organisms has a major influence on ecosystem dynamics and its functions (Lindeman 1942; Odum & Odum 1955; Parmelee 1995). A measure related to secondary production is the ratio of annual secondary production and mean annual biomass (P/B) which is used as an index of biomass turnover rate. Estimation of secondary production requires knowledge of population size, size-frequency distribution and length of developmental time. The first two components are normally assessed several times per year in the field, while developmental time is evaluated either from size-frequency distributions in the field or laboratory experiments (Huryn & Wallace 2000; Whalen & Parmelee 2000).

The effects on secondary production thus represent the influence of multiple stressors on general population performance and are useful for making inferences about the effects on food webs and ecosystem functioning. However, the impact on secondary production is determined by the partial effects on all its components: biomass, size-frequency distribution and length of developmental time or, related to it, body growth rate. The consequences of simultaneous action of multiple stressors for any of these population characteristics are difficult to predict, even knowing the reaction to these stressors acting separately. Therefore, in order to understand the mechanisms underlying the response of secondary production to multiple stressors, it is important also to analyse the responses of its components.

The soil fauna contributes significantly to energy and nutrient cycling and is responsible for regulating ecosystem functions like decomposition and nutrient mineralization (Hunt & Wall 2002; Heemsbergen *et al.* 2004; Fitter *et al.* 2005). It mediates directly and indirectly approximately 15% of carbon and 30% of nitrogen turnover with the highest contribution from those groups consuming fungal and bacterial biomass: protozoa and nematodes (Anderson 1995). Soil fauna composition

under stress usually differs from unstressed soil and the populations within the stressed community typically show altered characteristics (Yeates 2003; Ramsey *et al.* 2005). Contamination of soil with copper occurs in many areas across the world as a result of mining activities, sewage sludge disposal on agricultural fields and the use of copper-containing pesticides (Hopkin 1989). Although copper is essential for the normal function of cytochrome oxydase, high concentrations are toxic (Depledge *et al.* 1998). Negative effects of copper have been shown for many soil-inhabiting species as well as whole soil communities (Calow 1994; Korthals *et al.* 1996a). Acidification is another stress factor that is known to affect aquatic and soil systems (Calow 1994) and, currently, it is considered to be one of the major problems for ecosystems in Europe (EEA 2003). Soil acidification is accelerated by anthropogenic activities such as acidic deposition from air pollution and an imbalance in agricultural practices (Reuss & Johnson 1986; Cregan & Scott 1998).

In our study we used a bacterial-feeding, free-living nematode *Acrobeloides nanus* de Man 1880 (Nematoda, Cephalobidae). We analysed populations of *A. nanus* inhabiting six selected field treatments within a 20-year-old experimental field: three levels of pH (4.0; 4.7; 6.1) were combined with two levels of copper (0 and 750 kg ha^{-1}). The objective of our study was twofold: first, to investigate the influence of both stressors and their possible interaction on yearly secondary production and biomass turnover in the populations of *A. nanus* in the experimental field; secondly, to evaluate the effects of these stressors on biomass, size-frequency distribution, body growth rate and the contribution of these parameters to the changes in the level of P and P/B. Our aim was to detect general patterns in the response to multiple stressors. The temporal and spatial dynamics of the investigated parameters were beyond the scope of this study.

Materials and Methods

Study species

Acrobeloides nanus is a soil-dwelling, bacterial-feeding nematode that reproduces by parthenogenesis (Laugsch & Schierenberg 2004). The adult *A. nanus* is approximately half a millimetre long ('nanus' means 'dwarf'). *Acrobeloides nanus* commonly forms part of the nematode community in soils from different parts of the world, where it occupies a broad range of soil habitats and often constitutes as much as 20% of nematode communities (Bird & Ryder 1993; De Goede & Bongers 1998). *Acrobeloides nanus* feeds on saprophytic and plant pathogenic bacteria and contributes indirectly to organic matter decomposition by dispersing bacteria and viruses through the soil (Ikonen 2001). Under laboratory conditions at 20 ºC the generation time is

approximately 14 days and during the reproductive period approximately 250 eggs are laid per nematode.

Experimental protocol

The experimental field at Bovenbuurt, c. 3 km north-north-east of Wageningen, the Netherlands, was created in 1982, when an agricultural field was divided into 128 plots of 6 × 11 m each. The plots were arranged into 8 blocks, 22 × 48 m. Four pH and copper levels were introduced by applying $CuSO_4 \cdot 5H_2O$, sulphur powder or ground calcitic limestone, respectively in a full factorial design. All combinations of the copper and pH treatments were randomly distributed within each of the 8 blocks. In this study, six treatments were used, each represented by four replicate plots: treatments without copper addition combined with pH 4.0, 4.7 and 6.1 and treatments with the highest copper concentration of 750 kg Cu ha^{-1} combined with pH 4.0, 4.7 and 6.1. Hereafter we will use the following notation for the combined field treatments; for example, the treatment with no additional copper load and a pH of 4.0 is given as 'Cu0; pH 4.0', the treatment with a copper load of 750 kg ha^{-1} and a pH of 6.1 is given as 'Cu750; pH 6.1'. Maize, potatoes and oats have been grown in the field since 1983 in a 3-year crop rotation (although recently oats have been replaced by barley). The level of pH was readjusted every 5 years on average. Further information is given in (Korthals *et al.* 1996a). The present concentrations of copper and pH for four out of the six treatments used are shown in (Tobor-Kaplon *et al.* 2006). Although the actual pH values were lower than the experimentally aimed 'target' values, the relative differences between the treatments are maintained.

Nematode populations were sampled in April, July and September 2003. Approximately 30 cores (diameter 30 mm) from the top 10-cm soil layer were collected from each plot and mixed together. Subsequently, the nematodes were extracted according to a standard procedure (Oostenbrink 1960). A part of each sample was used to derive laboratory cultures of *A. nanus* for estimation of body growth rate and developmental time. The remaining parts of the samples were fixed with 4% formaldehyde.

Laboratory populations

Acrobeloides nanus was isolated from the extracted nematode samples with the aid of a microscope. Approximately 50 individuals from each plot were used to establish representative samples of field populations. Populations were cultured on Petri dishes with proteose peptone agar medium (PPA; 2% technical agar, 0.5% proteose peptone) and *Acinetobacter johnsonii* strain 210 A (the Netherlands Culture Collection of Micro-

organisms, access number LMAU A130, (Bonting *et al.* 1992), a soil bacterium used as a food source until the assessment of the body growth rate started (three generations).

Biomass and secondary production

The estimation of biomass was based on the density of *A. nanus* and body mass measurements. Densities were obtained by assessing the numbers of individuals of *A. nanus* in 100 g of soil while body mass measurements (fresh weight) were derived by recalculating nematode body length and width with Andrassy's formula (Freckman 1982):

$$W = (w^2 L)/(1.6\times10^6) \qquad \text{eqn 1}$$

Where W is the fresh weight (μg), w is the width (μm) at the widest point and L is the total length of a nematode (μm). Approximately 30 measurements of body length and width of fixed nematodes were taken for each plot population at every sampling occasion. Dry mass was calculated using the relationship proposed by Petersen (Yeates 1979), where dry mass was equal to 20% of fresh mass.

Annual secondary production was estimated with the size-frequency method (Hynes & Coleman 1968; Hamilton & Hynes 1969; Benke 1979) which does not require synchronous development. Biomass turnover was calculated from the obtained production values. The size-frequency method for estimating secondary production assumes that the mean size distribution over a year represents an 'average cohort'. The data on body mass of *A. nanus* were thus used to construct average annual cohorts with seven size classes for each plot. Production of the average cohort was calculated as the sum of the production losses between each two size classes. To calculate annual production, production of the average cohort was multiplied by the number of generations according to the modification of the Hynes method for multivoltine populations proposed by Benke (1979):

$$P = \{\sum [(\triangle N_c \times \overline{W_c}) \times h]\} \times (12/\mathrm{CPI}) \qquad \text{eqn 2}$$

where P is annual secondary production, $\triangle N_c$ the decrease in density between two consecutive size classes, $\overline{W_c}$ the average body mass of individuals from two consecutive size classes, h the number of size classes and CPI the duration of developmental time in months.

Mean relative growth rate (MRGR) estimation

CPI values were estimated for the nematodes from each plot separately on the basis of the mean relative growth rate (MRGR) measurements in the native soil environment using a minicontainer system (Lenz & Eisenbeis 1998; Arts *et al.* 2004). The experiment was started by placing synchronized eggs (Emmons *et al.* 1979) of each experimental population of *A. nanus* into the minicontainers with defaunated soil mixed with 10 μL of *A. johnsonii* suspension. The soils originated from the same plots as the populations and were defaunated by exposure to 60 ºC for 3 h. The bacterial cells were concentrated by centrifugation for 10 minutes at 2500 rpm yielding a suspension with a final concentration of approximately 10^9 cells mL^{-1}. The experiment was performed in the laboratory at 20 °C in dark. Every 3 – 5 days, one minicontainer of each population was collected. The nematodes were extracted from the minicontainers (Van Bezooijen 1997), heat killed and fixed in 4% formaldehyde. The body length and the largest body width were measured for approximately 20 individuals per minicontainer using a microscope at 40x objective magnification. Fresh body weight was calculated with Andrassy's formula (Freckman 1982), and subsequently was used to estimate the MRGR (per day):

$$MRGR = (\ln W_2 - \ln W_1)/ (t_2 - t_1), \qquad \text{eqn 3}$$

where W_2 stands for the bodyweight (μg) at the end of the experiment, and W_1 stands for the bodyweight (μg) at the beginning of the experiment; $(t_2\text{-}t_1)$ is the time in days between the two measurements (Radford 1967).

After approximately 12 days from the beginning of the experiment, 40 μL of concentrated *A. johnsonii* suspension (applied protocol for concentration as described above) was applied to each minicontainer to prevent food shortage. In order to obtain standardized MRGR values, body mass measurements were used as follows for MRGR calculations: the measurements from the last minicontainer before food addition were taken to be the initial body mass and the first measurements taken after food addition the final measurement.

MRGR was corrected to comply with the yearly average temperature of soil, assumed to be 12 °C, using two methods. The first method was based on the relation proposed by Arrhenius (Kooijman 1993), using Arrhenius temperature for *A. nanus* according to Jager (2005). In the second method, MRGR was experimentally assessed for *A. nanus* on agar medium at 12 °C and 20 °C and the obtained ratio, $MRGR_{20°C}/MRGR_{12°C}$, was used for correction of the temperature effect. CPI values were estimated from the corrected MRGR obtained with the latter method using the body weight of newly hatched juveniles and the adult body weight with equation 3.

Although obtaining CPI values directly from the field measurements would be a more appropriate approach if the ultimate goal of the study was the estimation of

secondary production and biomass turnover under field conditions, we chose laboratory estimates of MRGR to derive CPI values. Our decision was influenced by the fact that *A. nanus* CPI cannot be estimated from temporal patterns of size-frequency distributions in the field because of unidentifiable cohorts. Life-history is extremely flexible in this species and generation time can be as short as 10 days under favourable conditions or as long as several months or even years if a nematode enters a anabiotic stage under extremely unfavourable conditions. Further, in the experimental estimation of CPI we attempted to replicate field conditions by the use of soil from experimental plots, so that the populations' MRGR could be assessed in their native soils and the differences in this parameter could than be attributed to the imposed pH and copper treatments in the soil.

Data analysis

Acrobeloides nanus biomass was transformed by natural logarithm and production by quadratic root function to normalize the distributions. Secondary production and biomass turnover and their components were analysed with two-way ANOVA, with pH and Cu as fixed factors. As the effects of the treatments on size-frequency distribution could not be estimated directly, ANOVA for this trait was performed on the residuals derived after subtracting the variance associated with MRGR and biomass from the total variance of secondary production (Sokal & Rohlf 1995). All analyses were performed with SPSS 12.0.1.

Results

Effects on P and P/B

Secondary production was significantly affected by soil pH (Table 1). Low pH had a negative influence on production compared to the intermediate pH treatment (Fig. 1a; post-ANOVA Tukey test, $P = 0.007$). Although the overall copper effect was insignificant, ANOVA indicated a significant interaction between copper and pH (Table 1). The populations from the plots of pH levels 4.7 and 6.1 combined with high copper concentrations showed higher production than the populations of equivalent pH treatments without copper addition. The populations in the plots of low pH and high copper load showed the lowest production values (on average more than 50% lower than with the other treatments) indicating synergistic effects of these stressors. An example of the production calculation is presented in Table 2.

The average values of biomass turnover ranged from 12.6 for the plots of the treatment Cu750; pH 4.0 to 72 for the treatment Cu750; pH 4.7 (Fig. 1b). In general, the pattern of dependence of P/B on pH and copper followed that described for

secondary production (Fig 1a,b). P/B was significantly reduced in the plots of pH 4.0 (Table 1) in comparison with the two other pH treatments (post-ANOVA Tukey test, $P < 0.001$ and $P = 0.022$ for the comparisons with pH 4.7 and pH 6.1, respectively). As shown for production, the interaction between copper and pH was significant while no effect of copper was detected. Copper influenced P/B positively at pH 4.7 and 6.1 and negatively at pH 4.0.

Table 1: Summary of the ANOVA results for secondary production, biomass turnover, biomass, MRGR and size-frequency of A. nanus populations. All population parameters tested (except MRGR) were based on all three sampling events in 2003.

Response variable	Source of variation	df	*F*	*P*
Secondary production	copper	1	2.920	0.105
	pH	2	6.202	0.009
	copper × pH	2	9.393	0.002
	error	18		
Biomass turnover	copper	1	0.758	0.395
	pH	2	12.300	<0.001
	copper × pH	2	12.008	<0.001
	error	18		
Biomass	copper	1	6.118	0.024
	pH	2	0.329	0.724
	copper × pH	2	3.197	0.065
	error	18		
MRGR	copper	1	0.758	0.390
	pH	2	7.959	0.003
	copper × pH	2	7.233	0.005
	error	18		
Size structure	copper	1	0.042	0.841
	pH	2	2.262	0.133
	copper × pH	2	3.770	0.043
	error	18		

Effects on the components of P and P/B

In contrast to the results obtained for secondary production and biomass turnover, the biomass of *A. nanus* was affected only by copper (Table 1). Populations from the plots of higher copper load showed on average higher biomasses (Fig. 1c). The biomass ranged from 2.7 mg dry weight (dw) m^{-2} for the plots of the treatment Cu0; pH 4.7 to 6.5 mg dw m^{-2} for the treatment Cu750; pH 4.7.

The size-frequency distribution of the populations of *A. nanus* showed no effect of pH or copper treatment (Table 1). The interaction term pH × copper was significant, however the interpretation of this result is difficult.

Table 2: *Example of secondary production calculation for one of the A. nanus replicate populations of the treatment Cu0; pH 6.1 obtained with the size-frequency method*

Weight class (µg FM)	Density (No. m^{-2} 10^3) N	Individual mass (µg fw) W	No. lost (No. m^{-2} 10^3) N	Biomass (mg fw m^{-2}) $\Delta N \times W$	Mass lost (µg fw) $\overline{W_c}$	Biomass lost (mg fw m^{-2}) $\Delta N_c \times \overline{W_c}$	Biomass lost × No. of weight classes (mg fw m^{-2}) $\Delta N_c \times \overline{W_c} \times h$
0-0.039	127.2	0.029	22.44	3.69	0.0395	0.886	6.21
0.04-0.059	104.7	0.05	52.37	5.24	0.06	3.142	22.00
0.06-0.079	523.7	0.07	22.44	3.67	0.08	1.796	12.57
0.08-0.099	299.3	0.09	22.44	2.69	0.01	2.244	15.71
0.1-0.119	74.8	0.11	7.48	0.82	0.0615	0.460	32.21
0.12-0.139	0	0.013	0	0	0.965	0	0
0.14-larger	0	0.18	0	0	0.09	0	0

Annual mean biomass = $\sum(\Delta N \times W)$ = 16.11 mg fw m^{-2} = 3.22 mg dw m^{-2}

Cohort production (CP) = $\sum(\Delta N_c \times \overline{W_c} \times h)$ = 59.71 mg fw m^{-2}

Annual production (CP)×(12/CPI) = 59.71 mg fw m^{-2} × 6.9 = 412.2 mg fw m^{-2} = 82.44 mg dw m^{-2}

Annual P/B = 25.6

The temperature correction with the Arrhenius method resulted in approximately 50% higher MRGR compared with the estimates obtained from laboratory experiment. The method applied did not influence the general pattern of the dependence of MRGR on stress treatments. MRGR was affected by pH and the interaction between pH and copper. However, no significant effect of copper was detected (Table 1). The most adverse environment was treatment Cu750; pH 4.0 (Table 3 and Fig. 1d). The daily increase in fresh weight (fw) under these soil conditions was 0.012 μg, equivalent to a generation time of 16.6 months at 12 °C. This gives approximately a six-fold reduction compared with the most favourable environment Cu750; pH 4.7 where the daily increase in fw was 0.067 μg and generation time was 0.8 months at 12 °C.

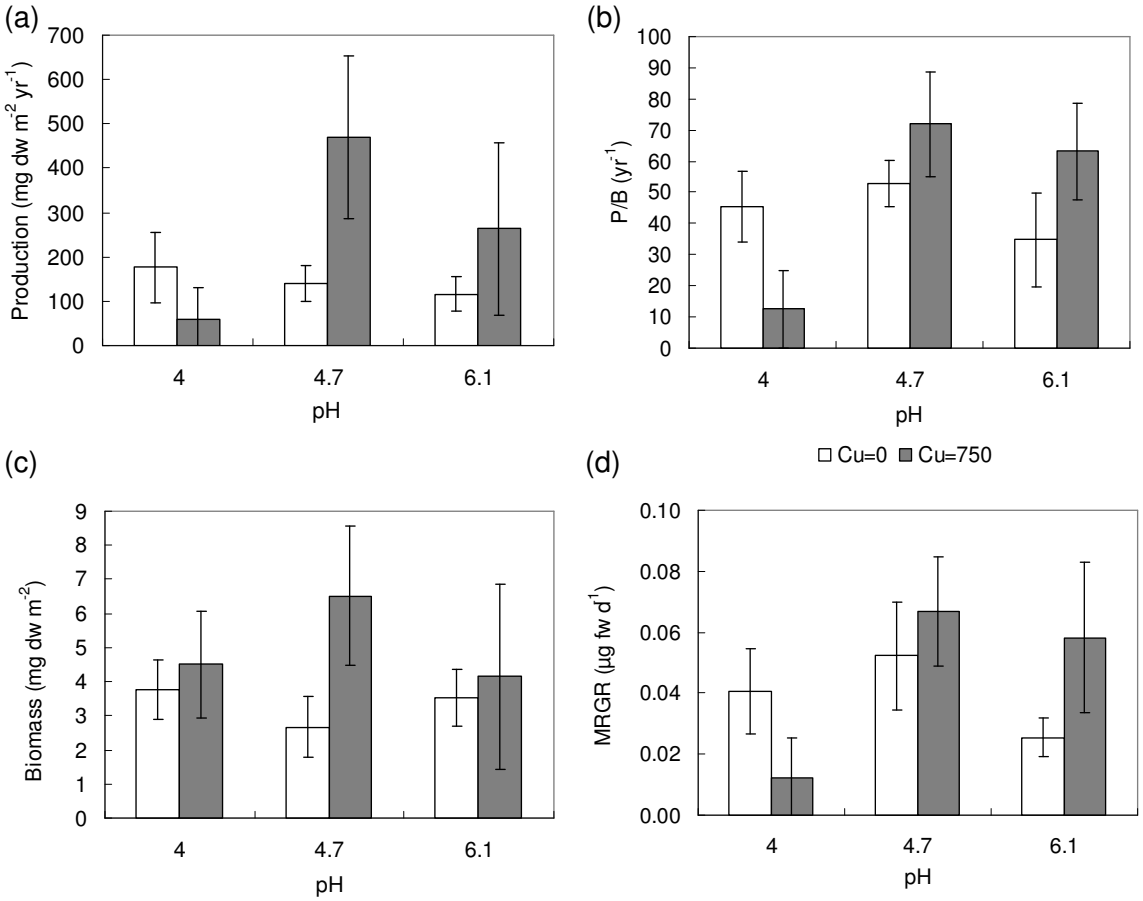


Figure 1: Secondary production (a), biomass turnover (b), biomass (c) and MRGR (d) of the populations of A. nanus (mean ± 1 SD; n replicate populations = 4) in different pH (4.0, 4.7, 6.1) and copper (0 kg Cu ha^{-1}; 750 kg Cu ha^{-1}) treatments. MRGR was assessed in the laboratory using the soil from the experimental plots. All population parameters (except MRGR) were based on all three sampling events in 2003.

Table 3: Summary of the descriptive statistics of MRGR and CPI values for the populations of A. nanus in their native soils at 12 ºC. The values obtained with temperature correction using Arrhenius relation and experimentally assessed relation are presented. Means ± SD are shown.

	MRGR (µg fw day^{-1})		CPI (months)	
Field treatment	Arrhenius	Experimental	Arrhenius relation	Experimental
Cu = 0 kg ha^{-1}				
pH 4.0	0.065 ± 0.022	0.041 ± 0.014	0.8 ± 0.39	1.1 ± 0.56
pH 4.7	0.083 ± 0.028	0.052 ± 0.018	0.8 ± 0.40	1.1 ± 0.60
pH 6.1	0.041 ± 0.010	0.025 ± 0.006	1.3 ± 0.66	1.8 ± 0.97
Cu = 750 kg ha^{-1}				
pH 4.0	0.019 ± 0.021	0.012 ± 0.013	12.2 ± 11.05	16.6 ± 15.87
pH 4.7	0.107 ± 0.029	0.067 ± 0.018	0.6 ± 0.31	0.8 ± 0.44
pH 6.1	0.093 ± 0.039	0.058 ± 0.025	0.7 ± 0.34	0.9 ± 0.50

In general, the response of MRGR to copper and pH resembled that of secondary production and biomass turnover. Regression analysis showed that the variance in MRGR explained 72.1% of the variance in secondary production. At the same time, 82.6% of P/B variance was explained by the variance in MRGR, which indicated the major role of the body growth rate in shaping the response of production and P/B to copper and pH in *A. nanus*.

Discussion

In this study we have demonstrated that the secondary production and biomass turnover of *A. nanus* increased when a higher copper load was combined with higher pH levels (pH 4.7 and 6.1) but the same copper load caused a substantial decrease in secondary production and biomass turnover when combined with pH 4.0 (approximately two-fold reduction in P and 3.5 fold reduction in P/B compared with the same pH treatment without copper addition).

To our knowledge this is the first attempt at investigating the individual and interactive effects of multiple stressors on secondary production, biomass turnover and their components using a full factorial experimental design in the field. The existing studies focusing on a single anthropogenic stressor usually report a decrease in both parameters under chronic stress. For example, higher concentrations of zinc and cadmium were found to negatively affect secondary production of freshwater invertebrates (Carlisle & Clements 2003; Perceval *et al.* 2004). Other authors (Lugthart & Wallace 1992; Whiles & Wallace 1995) showed that insecticide application reduced the production of macroinvertebrates inhabiting headwater streams. Although most studies demonstrate a decrease in secondary production and biomass turnover, some investigations have demonstrated that certain stressors may actually lead to their increase. Eutrophication and drought, for example, were found to enhance production (Lugthart & Wallace 1992; Shieh *et al.* 2002, 2003), and trawling disturbance had a positive effect on yearly biomass turnover while bringing no change in secondary production (Jennings *et al.* 2001). Currently, there are no comparable field data for nematodes. Existing laboratory studies have demonstrated synergistic effects of multiple stressors on some of the traits relevant for secondary production and biomass turnover rate. For example, some combinations of different heavy metals caused negative synergistic effects on survival in *Caenorhabditis elegans* (Chu & Chow 2002). In the same species body size was synergistically affected by copper-cadmium and copper-carbendazim mixtures (Jonker *et al.* 2004). Synergistic effects of a copper-cadmium mixture were also demonstrated in relation to reproduction of *Mesorhabditis monhystera* (current name *Bursilla monhystera*) and *Aphelenchus avenae* (Doelman *et al.* 1984).

In the present study the analysis of functional population parameters was performed for a single nematode species, *A. nanus*, which is known to maintain relatively high numbers compared with other nematode species in stressed soils and whose overall stress resistance is also considered to be relatively high. Therefore, more sensitive species that, unlike *A. nanus*, experience a biomass decrease under single or multiple stressors, are likely to display more severe responses of functional parameters under stress. The possible resistance of *A. nanus* should also be considered, as the measurements and parameter estimations reported here relate to sampling events approximately 20 years after the treatments were applied. The detailed study of life-history parameters of the populations from two extreme treatments Cu0; pH 6.1 and Cu750; pH 4.0, with the use of reaction norm and reciprocal transplant experiments, indicated adaptation to the conditions imposed by the treatments (chapter 3, this thesis).

One of the possible explanation for the synergistic effect of the stressors on secondary production and biomass turnover observed in our study is increased bioavailability of copper in soil of low pH. Soil pH is recognized as an important parameter influencing metal bioavailability and therefore also its toxicity for soil invertebrates (Van Gestel *et al.* 1995). The organic matter content, which is also considered one of the major determinants of metal availability, was similar across all analysed field treatment (M. A. Tobor- Kaplon, personal comunication) and therefore does not help explain the observed pattern.

Secondary production is determined mainly by biomass and body growth rate, hence any environmental factor that affects those parameters would also affect production (biomass turnover depends mostly on the body growth rate). Our results showed that MRGR was reduced in *A. nanus* grown in acidic soil (pH 4.0), with even stronger negative effects when low pH was combined with a high copper load (750 kg ha^{-1}). Inhibition of the growth rate and an increase in developmental time are common reactions to stress, thought to relate to lower food quality or quantity or increased energetic expenses required for the repair or defence mechanisms (Antonovics *et al.* 1971; Campbell & Grime 1992). The opposite responses of growth rate and developmental time have also been reported. Individuals may develop faster to avoid cumulative effects of the stressor (Stanton *et al.* 2000). Previous investigations of the effects of multiple stressors, conducted mainly on amphibian species, have demonstrated strong adverse effects on individual growth and development resulting from synergistic interaction between stressors (Chen *et al.* 2004; Boone *et al.* 2005).

Population biomass usually declines in response to stress; however, some populations may show no change or even an increase in biomass, which is often explained as a result of indirect effects such as reduced predation and competition (Morin 1999). Less is known about the effects of multiple stressors on population biomass, mainly because of a lack of replicated field experiments. Circumstantial

evidence suggests that negative effects may be exacerbated (Blaustein & Kiesecker 2002; Pandolfi *et al.* 2005). Our study, however, showed no interactive effects of the stressors. The only significant positive effect of copper may have appeared as a consequence of indirect effects, namely the effects on the community composition and functions. Copper could have a greater negative effect on predators or competing species, therefore releasing the pressures caused by these negative interactions. It has been shown that some predatory species, for example *Clarcus* and *Prionchulus* species, can be more sensitive to heavy metals and other stressors than most nematode species feeding on bacteria and fungi (Kammenga *et al.* 1994; Korthals *et al.* 1996b). Our study indicated relatively high sensitivity of these genera to the combination of copper load and low pH levels (data not shown). In general, it has been recognized that, under stress, positive relations between species are more common and populations experience less pressure from predators and, depending on the magnitude of stress, variable levels of competition (Morin 1999).

The pattern of MRGR response was similar to the response of production and biomass turnover. At the same time, biomass responded to the stressors in an entirely different way to the other parameters, indicating that biomass had a minor role in determining the response of P and P/B. These findings are in contrast to the majority of reported results, where secondary production was biomass driven (Carlisle & Clements 2003; Perceval *et al.* 2004). Nevertheless, some authors emphasize that body growth rate and environmental factors influence this trait. The results presented by Lugthart & Wallace (1992) and by Shieh *et al.* (2003) suggest that the effects of a particular stressor on biomass, abundance and production may differ. The effects on abundance and biomass may underestimate the effect of stress and therefore should be interpreted with caution.

The chosen size-frequency method requires quantification of CPI (duration of developmental time in months). CPI values were estimated on the basis of MRGR, which assumes that body growth is stable in all size classes. Such an approach can potentially lead to overestimation of secondary production and biomass turnover because large size classes have in fact lower growth rates but make up much of the population biomass (Huryn 1990). This effect, however, is not likely to bias the measurements to a serious degree because the development of *A. nanus* is fast and there was no accumulation of the higher size-classes. During the MRGR experiment, water content and soil compaction were optimized at the same level for all populations. Also predators and competitors were removed from close proximity to the nematodes tested. This protocol is likely to result in higher values of MRGR, and as a consequence, higher P and P/B compared with the field situation. Under field conditions, food may be limiting and body growth may be interrupted when individuals enter resistant juvenile stages. Therefore, application of the laboratory estimation of MRGR, where the food source was abundant, could potentially lead to

overestimation of secondary production and biomass turnover rate. In addition, the absence of the resistant stage during the MRGR experiment could possibly result in a more pronounced response of MRGR and consequently of CPI to the imposed stress compared with field conditions. Regardless of these limitations, the results of our experiment can be regarded as indicative of the response of MRGR to abiotic conditions, as the observed response could be attributed to the differences in soil properties defined by the experimental manipulation (copper load and soil pH).

For the calculation of CPI the yearly average temperature was assumed to be 12 ºC and temporal changes in production were not investigated. Certainly yearly average temperature changes from year to year and periods of low temperatures and heat can influence yearly production. Nevertheless, because the focus of this work was directed towards detecting the general pattern of stress response of functional population parameters and not their temporal dynamics, our approximation is appropriate. Additional estimates of P, P/B and their components were obtained for the same populations in the year 2002. The analysis indicated on average 30% lower biomass than in 2003 but the pattern of dependence on the treatment was similar: copper had a positive effect on biomass (ANOVA, $F = 3.80$, $P = 0.066$) with no apparent effect of pH or interaction between copper and pH (ANOVA, $F = 0.80$, $P = 0.46$ and $F = 0.98$, $P = 0.39$, respectively). Secondary production and biomass turnover were calculated using the same CPI values as in 2003 and therefore the calculations for these two consecutive years cannot be treated as independent. Nevertheless, secondary production was on average 50% and biomass turnover 20% lower in 2002 compared with 2003. The differences in these parameters between the years can probably be explained by the change in the crop type (barley in 2002 and potatoes in 2003) as well as the difference in weather conditions. Higher average temperatures of June and September of 2003 compared with the corresponding periods of 2002 were likely to have contributed to higher biomasses of *A. nanus*. These changes were parallel with the changes in the biomass of the whole nematode community in the years analysed (data not shown).

Our estimates of secondary production of *A. nanus* ranged from 0.29 to 2.82 kcal m^{-2} $year^{-1}$ (1.21-11.80 kJ m^{-2} $year^{-1}$). For the recalculations we assumed the caloric equivalent of 6 cal mg^{-1} dw and dry weight matter content of 20% based on several published results (Marchant & Nicholas 1974; Nicholas & Stewart 1978; Yeates 1979). In general, the reported estimates of secondary production for entire nematode communities fall roughly into range of 0.23-25.3 kcal m^{-2} $year^{-1}$ (0.96-105.88 kJ m^{-2} $year^{-1}$) depending on the habitat and the method of estimation (Yeates 1979). As in this study *A. nanus* constituted 2-25% of the nematode community, we can conclude that our estimates of secondary production for *A. nanus* populations fall into the range of reported values.

Previous investigations into the patterns of biomass turnover in soil organisms are inconclusive. While some authors suggest that low turnover is an intrinsic property of small soil-living animals including nematodes (Van Straalen 1989), others point to the fact that many small soil organisms have a short generation time that should result in higher values of P/B (Anderson 1995). The most often reported values of P/B for nematodes are close to 10 (Popovici 1984; Sohlenius *et al.* 1988; Verschoor 2002). In contrast, the populations of *A. nanus* in this study showed relatively high biomass turnover (approximately 60, range 12.1-71.9). Although these values are exceptionally high for a soil-inhabiting nematode, there are examples of marine nematodes feeding on bacteria with biomass turnover higher than 60 (Vranken & Heip 1986).

Despite a growing awareness of the variety of combined effects caused by multiple stressors, current risk assessment practices focus predominantly on single stressor effects. This is mainly a consequence of a poor general framework for multiple stressor risk assessment. Our results show that the reduction in production and biomass turnover rate was driven mainly by inhibition of body growth rate. This suggest that implementing analysis of this trait in laboratory toxicity tests of combined stressors in realistic concentrations can indicate potential risk for population functions. Our study also indicated that combined stressors can have a higher impact on functional population parameters than on biomass. This should be taken into account during development of regulatory policies directed towards conservation of functional properties of ecosystems and their key components.

Acknowledgements

We thank two anonymous reviewers and A.W.G. van der Wurff for helpful comments on the manuscript, and K. Ilieva-Makulec and H.G.C. Hilberink for technical help. This research was funded by the TRIAS Programme (TRIpartite Approach to Soil system processes) of Delft Cluster, SKB (Foundation for development and transmission of soil knowledge) and NWO (the Netherlands Organisation for Scientific Research), as project 835.80.008.

References

Anderson, J.M. (1995) Soil organisms as engineers: microsite modulation of macroscale processes. *Linking species and ecosystem* (eds C.G. Jones & J.H. Lawton), pp. 94-106. Chapman & Hall, New York.

Antonovics, J., Bradsham, A.D. & Turner, R.G. (1971) Heavy metal tolerance in plants. *Advances in Ecological Research*, **7**, 1–85.

Arts, M.S.J., Schill, R.O., Knigge, T., Eckwert, H., Kammenga, J.E. & Kohler, H. (2004) Stress proteins (hsp70, hsp60) induced in isopods and nematodes by field exposure to metals in a gradient near Avonmouth, UK. *Ecotoxicology*, **13**, 739-756.

Benke, A.C. (1979) Modification of the Hynes method for estimating secondary production with particular significance for multivoltine populations. *Limnology and Oceanography*, **24**, 168-171.

Bird, A.F. & Ryder, M.H. (1993) Feeding of the nematode *Acrobeloides nanus* on bacteria. *Journal of Nematology*, **25**, 493-499.

Blaustein, A.R. & Kiesecker, J.M. (2002) Complexity in conservation: lessons from the global decline of amphibian populations. *Ecology Letters*, **5**, 597-608.

Bonting, C.F.C., Willemsen, B.M.F., Akkermans - Van Vliet, W., Bouvet, P.J.M., Kortstee, G.J.J. & Zehnder, A.J.B. (1992) Additional characteristics of the polyphosphate-accumulating *Acinetobacter* strain 210a and its identification as *Acinetobacter johnsonii*. *FEMS Microbiology Ecology*, **102**, 57-64.

Boone, M.D., Bridges, C.M., Fairchild, J.F. & Little, E.E. (2005) Multiple sublethal chemicals negatively affect tadpoles of the green frog, *Rana clamitans*. *Environmental Toxicology and Chemistry*, **24**, 1267-1272.

Breiburg, D.L., Baxter, J.W. & Hatfield, C.A. (1998) Understanding effects of multiple stressors: ideas and challenges. *Successes, limitations and frontiers in ecosystem science* (eds M.L. Pace & P.M. Groffman), pp. 416-431. Springer-Verlag, New York.

Calow, P., ed. (1994) *Handbook of ecotoxicology*, 2nd edn. Bleckwell Science, Oxford, UK.

Campbell, B.D. & Grime, J.P. (1992) An experimental test of plant strategy theory. *Ecology*, **73**, 15-29.

Carlisle, D.M. & Clements, W.H. (2003) Growth and secondary production of aquatic insects along a gradient of Zn contamination in Rocky Mountain streams. *Journal of the North American Benthological Society*, **22**, 582-597.

Caswell, H. (1989) *Matrix population models* Sinauer Associates, Sunderland, Massachusetts.

Chen, C.Y., Hathaway, K.M. & Folt, C.L. (2004) Multiple stress effects of Vision (R) herbicide, pH, and food on zooplankton and larval amphibian species from forest wetlands. *Environmental Toxicology and Chemistry*, **23**, 823-831.

Chu, K.W. & Chow, K.L. (2002) Synergistic toxicity of multiple heavy metals is revealed by a biological assay using a nematode and its transgenic derivative. *Aquatic Toxicology*, **61**, 53-64.

Cregan, P.D. & Scott, B.J. (1998) Soil acidification-an agricultural and environmental problem. *Agriculture and environmental imperative* (eds J.E. Pratley & A. Robertson), pp. 98-128. CSIRO, Melbourne.

De Goede, R.G.M. & Bongers, T. (1998) *Nematode communities of northern temperate grassland ecosystems* Focus, Giessen.

Depledge, M.H., Weeks, J.M. & Bjerregaard, P. (1998) Heavy metals. *Handbook of ecotoxicology* (ed P. Calow), pp. 543-569. University Press, Cambridge.

Doelman, P., Nieboer, G., Schrooten, J. & Visser, M. (1984) Antagonistic and synergistic toxic effects of Pb and Cd in a simple foodchain - nematodes feeding on bacteria or fungi. *Bulletin of Environmental Contamination and Toxicology*, **32**, 717-723.

EEA (2003) *Europe's environment: the third assessment.* European Environmental Agency, Copenhagen.

Emmons, S.W., Klass, M.R. & Hirsh, D. (1979) Analysis of the constancy of DNA sequences during development and evolution of the nematode *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences, USA*, **76**, 1333-1337.

Fitter, A.H., Gilligan, C.A., Hollingworth, K., Kleczkowski, A., Twyman, R.M. & Pitchford, J.W. (2005) Biodiversity and ecosystem function in soil. *Functional Ecology*, **19**, 369-377.

Freckman, D.W. (1982) Nematode contributions to ecosystem. *Nematodes in Soil Ecosystems* (ed D.W. Freckman), pp. 81-97. University of Texas Press, Austin.

Hamilton, A.L. & Hynes, H.B.N. (1969) On estimating annual production. *Limnology and Oceanography*, **14**, 771-782.

Heemsbergen, D.A., Berg, M.P., Loreau, M., van Haj, J.R., Faber, J.H. & Verhoef, H.A. (2004) Biodiversity effects on soil processes explained by interspecific functional dissimilarity. *Science*, **306**, 1019-1020.

Hopkin, S.P. (1989) *Ecophysiology of metals in terrestrial invertebrates* Elsevier Applied Science Publishers, London.

Hunt, H.W. & Wall, D.H. (2002) Modelling the effects of loss of soil biodiversity on ecosystem function. *Global Change Biology*, **8**, 33-50.

Huryn, A.D. (1990) Growth and voltinism of lotic midge larvae - patterns across an Appalachian Mountain Basin. *Limnology and Oceanography*, **35**, 339-351.

Huryn, A.D. & Wallace, J.B. (2000) Life history and production of stream insects. *Annual Review of Entomology*, **45**, 83-110.

Hynes, H.B.N. & Coleman, M.J. (1968) A simple method of assessing annual production of stream benthos. *Limnology and Oceanography*, **13**, 569-&.

Ikonen, E.K. (2001) Life history studies of the hermaphrodite nematode *Acrobeloides* sp with *Pseudomonas cepacia* as a food source on agar. *Nematology*, **3**, 759-766.

Jager, T., Álvarez, O.A., Kammenga, J.E. & Kooijman, S. (2005) Modelling nematode life cycles using dynamic energy budgets. *Functional Ecology*, **19**, 136-144.

Jennings, S., Dinmore, T.A., Duplisea, D.E., Warr, K.J. & Lancaster, J.E. (2001) Trawling disturbance can modify benthic production processes. *Journal of Animal Ecology*, **70**, 459-475.

Jonker, M.J., Piskiewicz, A.M., Castella, N.I.I. & Kammenga, J.E. (2004) Toxicity of binary mixtures of cadmium-copper and carbendazim-copper to the nematode *Caenorhabditis elegans*. *Environmental Toxicology and Chemistry*, **23**, 1529-1537.

Kammenga, J.E., Van Gestel, C.A.M. & Bakker, J. (1994) Patterns of sensitivity to cadmium and pentachlorophenol among nematode species from different taxonomic and ecological groups. *Archives of Environmental Contamination and Toxicology*, **27**, 88-94.

Knowlton, N. (2001) The future of coral reefs. *Proceedings of the National Academy of Sciences, USA*, **98**, 5419-5425.

Kooijman, S.A.L.M. (1993) *Dynamic Energy Budgets in Biological Systems*, 1st edn. Cambridge University Press, Cambridge.

Korthals, G.W., Alexiev, A.D., Lexmond, T.M., Kammenga, J.E. & Bongers, A.M.T. (1996a) Long-term effects of copper and pH on the nematode community in an agroecosystem. *Environmental Toxicology and Chemistry*, **15**, 979-985.

Korthals, G.W., Van de Ende, A., Van Megen, H., Lexmond, T.M., Kammenga, J.E. & Bongers, T. (1996b) Short-term effects of cadmium, copper, nickel and zinc on soil nematodes from different feeding and life-history strategy groups. *Applied Soil Ecology*, **4**, 107-117.

Laugsch, M. & Schierenberg, E. (2004) Differences in maternal supply and early development of closely related nematode species. *International Journal of Developmental Biology*, **48**, 655-662.

Lenz, R. & Eisenbeis, G. (1998) An extraction method for nematodes in decomposition studies using the minicontainer-method. *Plant and Soil*, **198**, 109-116.

Lindeman, R.L. (1942) The trophic-dynamic aspect of ecology. *Ecology*, **23**, 399-418.

Lugthart, G.J. & Wallace, J.B. (1992) Effects of disturbance on benthic functional structure and production in mountain streams. *Journal of the North American Benthological Society*, **11**, 138-164.

Marchant, R. & Nicholas, W.L. (1974) Energy budget for free-living nematode *Pelodera* (*Rhabditidae*). *Oecologia*, **16**, 237-252.

Morin, P.J. (1999) *Community ecology* Blackwell Science, New Brunswick, New Jersey.

Nicholas, W.L. & Stewart, A.C. (1978) Calorific value of *Caenorhabditis elegans* (Rhabditidae). *Nematologica*, **24**, 45-50.

Odum, H.T. & Odum, E.P. (1955) Trophic structure and productivity of a windward coral reef community on Eniwetok Atoll. *Ecological Monographs*, **25**, 291-320.

Oostenbrink, M. (1960) Estimating nematode populations by some selected methods. *Nematology* (eds J.N. Sasser & W.R. Jenkins), pp. 85-102. The University of North Carolina Press, Chapel Hill, NC.

Pandolfi, J.M., Jackson, J.B.C., Baron, N., Bradbury, R.H., Guzman, H.M., Hughes, T.P., Kappel, C.V., Micheli, F., Ogden, J.C., Possingham, H.P. & Sala, E. (2005) Ecology - Are US coral reefs on the slippery slope to slime? *Science*, **307**, 1725-1726.

Parmelee, R.W. (1995) Soil fauna: Linking different levels of the ecological hierarchy. *Linking species and ecosystems* (eds C.G. Jones & J.H. Lawton), pp. 107-116. Chapman & Hall, New York.

Perceval, O., Couillard, Y., Pinel-Alloul, B., Giguere, A. & Campbell, P.G.C. (2004) Metal-induced stress in bivalves living along a gradient of Cd contamination: relating sub-cellular metal distribution to population-level responses. *Aquatic Toxicology*, **69**, 327-345.

Popovici, I. (1984) Nematode abundance, biomass and production in a beech forest ecosystem. *Pedobiologia*, **26**, 205-219.

Preston, B.L. (2002) Indirect effects in aquatic ecotoxicology: Implications for ecological risk assessment. *Environmental Management*, **29**, 311-323.

Radford, P.J. (1967) Growth analysis formulae - their use and abuse. *Crop Science*, **7**, 171-175.

Ramsey, P.W., Rillig, M.C., Feris, K.P., Gordon, N.S., Moore, J.N., Holben, W.E. & Gannon, J.E. (2005) Relationship between communities and processes; new insights from a field study of a contaminated ecosystem. *Ecology Letters*, **8**, 1201-1210.

Reuss, J.O. & Johnson, D.W. (1986) *Acid deposition and the acidification of soils and waters* Springer-Verlag, New York.

Salbu, B., Rosseland, B.A. & Oughton, D.H. (2005) Multiple stressors - a challenge for the future. *Journal of Environmental Monitoring*, **7**, 539-539.

Shieh, S.H., Ward, J.V. & Kondratieff, B.C. (2002) Energy flow through macroinvertebrates in a polluted plains stream. *Journal of the North American Benthological Society*, **21**, 660-675.

Shieh, S.H., Ward, J.V. & Kondratieff, B.C. (2003) Longitudinal changes in macroinvertebrate production in a stream affected by urban and agricultural activities. *Archiv Fur Hydrobiologie*, **157**, 483-503.

Sih, A., Bell, A.M. & Kerby, J.L. (2004) Two stressors are far deadlier than one. *Trends in Ecology and Evolution*, **19**, 274-276.

Sohlenius, B., Boström, S. & Sandor, A. (1988) Carbon and nitrogen budgets of nematodes in arable soil. *Biology and Fertility of Soils*, **6**, 1-8.

Sokal, R.R. & Rohlf, F.G. (1995) *Biometry* Freeman, New York.

Stanton, M.L., Roy, B.A. & Thiede, D.A. (2000) Evolution in stressful environments. I. Phenotypic variability, phenotypic selection, and response to selection in five distinct environmental stresses. *Evolution*, **54**, 93-111.

Tobor-Kaplon, M.A., Bloem, J. & de Ruiter, P.C. (2006) Functional stability of microbial communities from long-term stressed soils to additional disturbance. *Environmental Toxicology and Chemistry*.

Van Bezooijen, J. (1997) *Methoden en technieken voor nematologie*, Wageningen University and Research Centre, Wageningen.

Van Gestel, C.A.M., Rademaker, M.C.J. & Van Straalen, N.M. (1995) Capacity controlling parameters and their impact on metal toxicity. *Biogeodynamics of Pollutants in Soils and Sediments* (eds W. Salomons & W.M. Stigliani), pp. 171-192. Springer-Verlag, New York.

Van Straalen, N.M. (1989) Production and biomass turnover in two populations of forest floor collembola. *Netherlands Journal of Zoology*, **39**, 156-168.

Verschoor, B.C. (2002) Carbon and nitrogen budgets of plant-feeding nematodes in grasslands of different productivity. *Applied Soil Ecology*, **20**, 15-25.

Vranken, G. & Heip, C. (1986) The productivity of marine nematodes. *Ophelia*, **26**, 429-442.

Whalen, J.K. & Parmelee, R.W. (2000) Earthworm secondary production and N flux in agroecosystems: a comparison of two approaches. *Oecologia*, **124**, 561-573.

Whiles, M.R. & Wallace, J.B. (1995) Macroinvertebrate production in a headwater stream during recovery from anthropogenic disturbance and hydrologic extremes. *Canadian Journal of Fisheries and Aquatic Sciences*, **52**, 2402-2422.

Yan, N.D., Keller, W., Scully, N.M., Lean, D.R.S. & Dillon, P.J. (1996) Increased UV-B penetration in a lake owing to drought-induced acidification. *Nature*, **381**, 141-143.

Yeates, G.W. (1979) Soil nematodes in terrestrial ecosystems. *Journal of Nematology*, **11**, 213-229.

Yeates, G.W. (2003) Nematodes as soil indicators: Functional and biodiversity aspects. *Biology and Fertility of Soils*, **37**, 199-210.

Chapter 3

Rapid adaptive divergence of life-history traits in response to abiotic stress within a natural population of a parthenogenetic nematode

Agnieszka Doroszuk
Marcin W. Wojewodzic
Jan E. Kammenga

2006, *Proceedings of the Royal Society of London (B), 273 (1601): 2611-2618*

Abstract

Sexual reproduction is acknowledged to facilitate adaptation to novel environments while asexual eukaryotes are often regarded as having low adaptive potential. This view has been challenged in a number of studies, but the adaptive potential of asexual populations in the field is poorly documented. We investigated the response of natural populations of the parthenogenetic nematode *Acrobeloides nanus* to imposed divergent selective pressures. For that purpose, we employed a replicated evolution experiment in the field. After 20 years of evolution under abiotic stress and control conditions, life-history traits were assessed in reaction norm- and in reciprocal transplant experiments. Both these experiments indicated adaptive divergence within the population of *A. nanus*. Namely, the transplant experiment demonstrated that in the stressed soil environment, body growth rate was more reduced in the nematodes originating from the control treatment. In the reaction norm experiment, survival and reproduction were higher under test conditions corresponding to the native environment of the nematodes. The differences in the analysed traits are discussed in the context of life-history theory. Overall, our results strongly support high adaptive potential of *A. nanus* and suggest that population structure and distribution of asexual species is shaped by local adaptation events.

Introduction

Asexual populations are expected to have lower adaptive potential than the corresponding sexual populations. This prediction follows the reasoning that the absence of recombination in asexuals results in a lower standing genetic variation available for selection (Goddard *et al.* 2005). In addition, without recombination, new beneficial mutations arising in different individuals cannot be combined to create more successful genotypes. Nevertheless, evidence from microevolutionary studies on bacteria and viruses shows that asexual populations are able to increase their fitness in novel environments without population depreciation or extinction (Travisano & Lenski 1996). Less rapid responses can be expected in eukaryotic populations reproducing asexually because of their lower mutation rates and smaller population sizes. The empirical studies appear to confirm this view by reporting very few cases of rapid adaptive evolution in natural populations of asexual eukaryotes (but see Dickson 1962). On the other hand, the absence of recombination restrains erosion of beneficial associations between loci. Therefore, populations are more likely to diverge and local adaptation, once arisen, can be easily maintained.

The first step in studying adaptive divergence involves the detection of phenotypic differences between local populations and testing the adaptive value of the observed changes. Usually, the local populations are tested in two habitats in a reciprocal transplant experiment and the relative fitness or fitness-related traits are compared. When the fitness of both population is higher in their native habitats, it can be concluded that the populations are locally adapted (Kawecki & Ebert 2004). The other type of experiment to detect local adaptation involves tests under controlled laboratory conditions, where only certain properties of the field habitat are recreated. Different local populations can, for instance, be reared under the same environmental conditions or be subjected to different levels of a specific environmental factor (reaction norm experiments). The advantage of the latter is that particular environmental factors can be tested as source of divergent selection.

Environmental stress, either of human or non-human origin, causes a sharp reduction in fitness and fitness-related traits and is therefore an important drive of evolutionary processes (Bijlsma & Loeschcke 2005). Numerous cases are documented where localized stress conditions led to adaptive divergence within populations (e.g. McNeilly 1968; Berglund *et al.* 2004). According to life-history theory, there are two possible scenarios of evolution of life-history traits in a stressful environment. Adverse effects of a stress agent often accumulate over life-time (e.g. concentration of heavy metals within the body), which can cause an increase in adult mortality (Posthuma & Van Straalen 1993). One of the possible evolutionary responses to this situation involves earlier onset of reproduction and higher reproduction at a younger age. In this way, the fitness effects of increased mortality later in life can be avoided (Stanton *et al.* 2000). Indeed, several studies indicated rapid development and early reproduction

as evolutionary response to long-term stress (Rice & Mack 1991; Aronson *et al.* 1992). The other possible scenario would involve investment in defence mechanisms like detoxification or excretion. As a result, an individual could enjoy higher adult survival. Because the energy invested in the defence cannot be used for body growth or reproduction, the onset of reproduction is delayed and the rate of offspring production is reduced. This scenario, compared to the previous one, has a broader empirical documentation (Antonovics *et al.* 1971; Grime & Hunt 1975; Campbell & Grime 1992; Donker 1992).

In our study we focused on a soil-dwelling, bacterivorous nematode *Acrobeloides nanus* de Man 1880 (Nematoda, Cephalobidae) that reproduces by means of parthenogenesis (e.g. Bird *et al.* 1993; Goldstein *et al.* 1998; Lahl *et al.* 2006). Egg development in *A. nanus* was described as essentially mitotic with rare cases of second meiotic division, where the restoration of diploidy occurs by the fusion of oocyte nucleus with second polar body (Lahl *et al.* 2006). Although few male individuals of *A. nanus* have been reported in two taxonomical studies (Nesterov 1979; Bongers 1988), validity of this record can be questioned not only because of the lack of mating test, but also because of uncertainty in correspondence of the morphological characteristics of the mails with species description of *A. nanus* (T. Bongers, S. Boström 2006, personal communication). Moreover, none of numerous laboratory and field studies of *A. nanus* recorded any males even after application of severe abiotic stresses which are known to induce emergence of males in non-obligate parthenogenetic species (Bird 1971; Triantaphyllou 1973).

Individuals of *A. nanus* can be found in soils of various physical and chemical properties (Bird *et al.* 1993; Korthals 1997; De Goede & Bongers 1998) and constitute a large part of soil nematode communities in extremely different habitats like the deserts of Australia (Bird et al. 1993) and Swedish tundra (Sohlenius & Bostrom 1999). Such broad species niche could be attributed to the possible existence of general purpose genotypes (Lynch 1984) or it could indicate multiple events of adaptation to the local environment (Futuyma & Moreno 1988). Therefore, *A. nanus* is a valuable biological system to investigate the potential for adaptive divergence in asexual species that successfully inhabit broad range of habitats. Showing that a population of *A. nanus* can undergo multiple events of local adaptation within a short time would indicate large adaptive potential that could be responsible for its broad distribution.

To address this question, we used an experimental field where combined treatments of two stress factors (pH level and copper) were applied approximately 20 years ago in a replicated and randomized block design and maintained thereafter. In that way, the population of *A. nanus* that inhabited the field before the treatment application was divided into a number of subpopulations exposed to different treatments of the stress factors. In order to investigate adaptation to the local environments and test the predictions of life-history theory concerning adaptation to

a stressful environment, we analysed life-history traits of the populations evolving for 20 years in control and stress conditions (low pH and high copper load) using reciprocal transplant and reaction norm experiments.

Material and methods

Experimental field and experimental populations

a) Study species

Acrobeloides nanus is a free living, bacterial-feeding nematode that reproduces by parthenogenesis (Wiegner & Schierenberg 1998; Laugsch & Schierenberg 2004). It can be easily cultured on plates with agar media seeded with bacteria. Under laboratory conditions at 20 ºC, the generation time is approximately 14 days and 250 eggs are laid during the reproductive period of 35 days. On the basis of previous experiments (A. Doroszuk, unpublished data) and the analysis of body growth rate in the transplant experiment, the number of generations per year under field conditions was estimated as 0.7 (14 generations in 20 years) for the 'stress' plots and 6.6 generations per year (132 generations in 20 years) for the control plots.

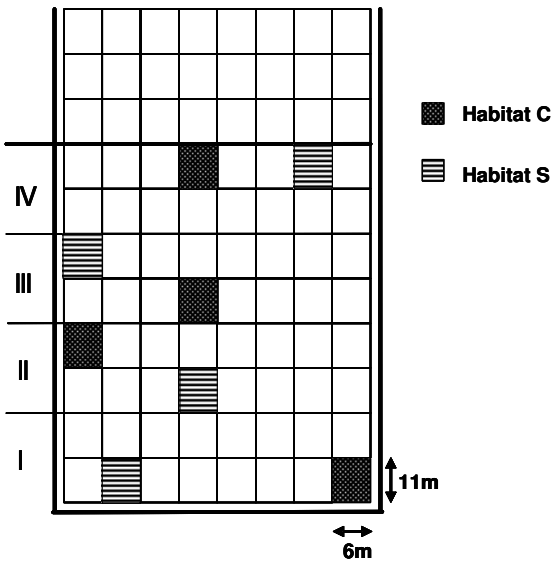


Figure 1: Distribution of treatments at the experimental field. Roman numbers indicate randomized blocks. habitat C represents control conditions (pH 6.1; Cu = 0 kg ha^{-1}); habitat S represents stressful conditions (pH 4.0; Cu = 750 kg ha^{-1}).

b) Sampling and field description

Nematodes were isolated, with standard techniques (Oostenbrink 1960), from soil samples collected from the experimental field at Bovenbuurt, c. 3 km north-north-east of Wageningen, the Netherlands (Korthals *et al.* 1996). The experimental field was created in 1982 when an agricultural field was divided into 128 plots of 6 × 11 m each and four copper and pH levels were introduced by applying once $CuSO_4 \cdot 5H_2O$,

sulphur powder or ground calcitic limestone, respectively, in a full factorial design. The plots were arranged into eight blocks, each with a random distribution of all combinations of copper and pH treatments. Only two extreme treatments were used (each represented by four replicate plots): one treatment imposing stress by low pH (4.0) and the copper concentration of 750 kg Cu ha^{-1} (habitat S) and another control treatment with pH 6.1 without copper addition (habitat C; Fig. 1). Maize, potatoes and oats have been grown on the field since 1983 in 3-year crop rotation regime. The pH was readjusted every 5 years on average.

About 30 cores (diameter 30mm) from the top 10 cm of soil mineral layer were taken from each of the plots. Nematodes classified as *A. nanus* were isolated and placed individually on the Petri dish with proteose peptone agar (PPA; 2% technical agar, 0.5% proteose peptone) medium and *Acinetobacter johnsonii*, a soil bacterium, as a food source in order to establish approximately 20 parthenogenetically derived laboratory populations per plot.

About 16-20 laboratory populations per plot were mixed to establish the representative samples of field populations (8 populations = 2 habitats × 4 replicate plots). The individuals of the fourth generation reared in the laboratory were used in all experiments.

Testing for adaptation

a) Reciprocal transplant experiment

Eight experimental populations (2 habitats × 4 replicate plots) were reciprocally transplanted between the soils of both habitats collected from the experimental field of Bovenbuurt. For each population, body growth rate expressed as mean relative growth rate (MRGR) was assessed. The equation for the MRGR is:

$$MRGR = \frac{(\ln W_2 - \ln W_1)}{(t_2 - t_1)} \qquad \text{eqn 1}$$

where W_2 stands for the bodyweight (μg) at the end of the experiment (t_2) and W_1 stands for the bodyweight (μg) at the beginning of experiment (t_1) (Radford 1967).

The experiment was started by placing synchronized eggs (Emmons *et al.* 1979) of each experimental population into minicontainers (Lenz & Eisenbeis 1998; Arts *et al.* 2004) with defaunated soil mixed with a suspension of soil bacteria *A. johnsonii* (2×10^8 cells mL^{-1} yeast extract). The soil was obtained from the plots of the populations' origin and was defaunated by exposure to 60 °C for 3 h. The experiment was performed in the laboratory at 20 °C in dark. Every 3-5 days, one minicontainer of each population was collected. The nematodes were extracted from the minicontainers (Van Bezooijen 1997), heat killed and fixed in 4% formalin. Body

length and largest body width were measured for approximately 20 individuals per minicontainer using a microscope at 40× magnification and with ocular equipped with a scale. The body length and width measurements were used to calculate body fresh weight with the use of Andrassy's formula (Freckman 1982). These measurements were used to calculate the MRGR per day.

After approximately 12 days from the start of the experiment, 40 μL of concentrated *A. johnsonii* suspension was applied to each minicontainer to prevent food shortage. In order to obtain standardized MRGR values, the following body mass measurements were used for MRGR calculations: the measurements from the last minicontainer before food addition as initial body mass and the measurements taken first after food addition as the final measurement. The MRGR data were analysed with a mixed-model nested ANOVA using PROC GLM procedure in SAS version 8.00 (SAS Institute 2000). Field treatment and soil environment were considered fixed factors and replicate plot was nested within the field treatment.

b) Reaction norm experiment: copper

For this experiment, 24-well dishes with PPA of six nominal copper concentrations: 0, 60, 100, 120, 150 and 180 mg $CuCl_2$ L^{-1} were used. The metal was added to the agar as $CuCl_2$ solution and 2 μL of suspension of *A. johnsonii* was applied. The dishes were incubated overnight at 28 ºC prior to use. Eggs of all eight experimental populations were synchronized according to Emmons *et al.* (1979) and transferred individually to separate wells. The experiment started with 12 eggs per population in each copper concentration (total N = 576 = 12 eggs × 8 populations × 6 concentrations). After hatching, the nematodes were transferred every other day to new wells. On the basis of daily observation, for each individual the duration of juvenile and reproductive periods, total number of offspring, daily reproduction and life span were determined. The dishes were incubated at 20 °C in dark.

Comparison of two field treatments in total and daily reproduction was done by comparing the values of EC_{50}, which were determined using the following logistic model:

$$E = \frac{\alpha}{1 + (x / EC_{50})^{\beta}} \qquad \text{eqn 2}$$

where E is trait value; a is the trait value at $x = 0$; x is copper concentration; EC_{50} is the concentration of copper that causes 50% effect; and β is the shape parameter of the curve. The curve was fitted using nonlinear regression procedure. Reproductive period was analyzed with the use of two-way ANOVA with field treatments and copper concentration as fixed factors. For the analysis of survival curves as well as

juvenile period, we implemented log-rank and Wilcoxon tests (SPSS 11.0). The data for the replicate populations within habitats were pooled.

c) Reaction norm experiment: pH

In order to obtain agar medium with pH values: 4.0, 4.5, 5.0, 5.5 and 6.0, citric buffer system in modified nematode growth medium (Wood 1988) was used. The osmolarity of the agar medium was kept constant by adjusting the concentration of NaCl. The experimental set-up, the order of consecutive actions and the life-history traits determined for each individual were the same as in the copper response experiment. The response of the life-history traits was analysed using two-way ANOVA with field treatment and pH level as fixed factors. For the analysis of survival curves as well as juvenile period, we implemented log-rank and Wilcoxon tests. All analyses were performed with the use of SPSS 11.0.

Results

Testing adaptation

a) Reciprocal transplant experiment

MRGR of the nematodes originating from the plots of both treatments was significantly lower in the soil of habitat S ($F = 39.45$, $P < 0.001$). However, the adverse effect was more severe for the populations from the plots of habitat C (Fig. 2).

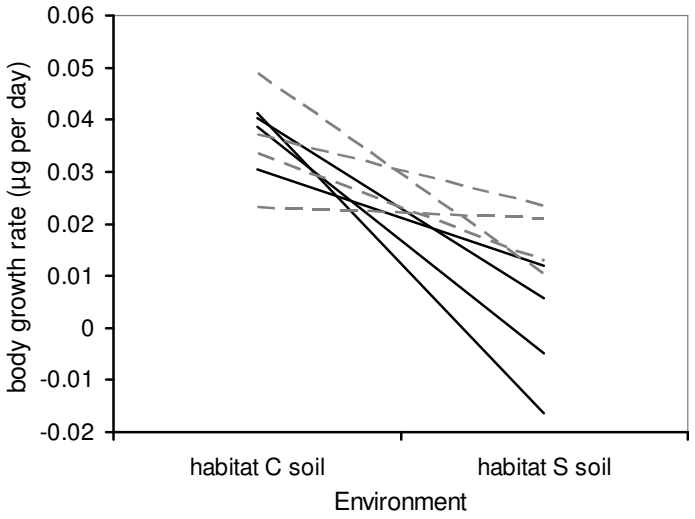


Figure 2: Mean relative growth rate (MRGR) of the experimental populations from habitat C (solid lines) and habitat S (dashed lines) tested undr two environmental conditions in a reciprocal transplant experiment. Each line connects MRGR values for one replicate population in two test environments.

This difference in populations' response to the soil environment was confirmed by significant interaction origin habitat × test environment ($F = 4.74$, $P = 0.05$).

b) Reaction norms experiment: copper

The populations derived from the plots of habitat S were less affected by copper in total reproduction (Fig. 3), which is reflected by the significantly higher value of EC_{50} for this trait (EC_{50} = 169.0 mg L^{-1}, Confidence Interval (CI): 162.5-175.5 mg L^{-1}) compared to the EC_{50} value obtained for the populations from habitat C (EC_{50} = 137.2 mg L^{-1}, CI: 132.8-141.5 mg L^{-1}). A similar pattern was obtained for daily reproduction where EC_{50} = 201.1 mg L^{-1} (CI: 191.0-211.1 mg L^{-1}) for the population from habitat S and EC_{50} = 156.8 mg L^{-1} (CI: 153.4-160.1 mg L^{-1}) for the populations from habitat C.

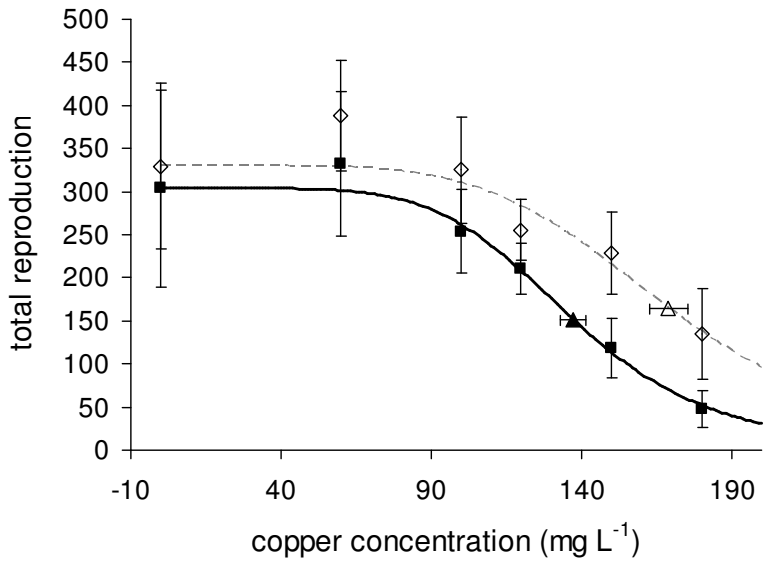


Figure 3: Response of total reproduction of A. nanus originating from the plots of habitat C (closed symbols, solid line, replicate populations pooled) habitat S (open symbols, dashed line, replicate populations pooled) to different concentrations of copper in the agar medium. Mean ± SD is shown. Values of EC_{50} for both pooled populations are indicated by the triangles (± CI).

Reproductive period was on average shorter in the populations from the plots of habitat C ($F = 17.87$, $P < 0.001$) and both populations reacted to the higher copper concentration with reduced reproductive period ($F = 122.00$, $P < 0.001$). Although the significant interaction habitat × copper concentration ($F = 5.423$, $P < 0.001$) indicates differences in the response patterns between the populations from different habitats, no conclusion regarding the difference in general tolerance to copper could be made (Fig. 4). In general, increase of copper concentration in the medium caused decrease in survival probability in populations of both treatments (for the populations from habitat S: log-rank test, $\chi^2 = 94.32$, $P < 0.001$; for the populations from habitat C: log-rank test, $\chi^2 = 192.34$, $P < 0.001$). The only deviation from this pattern was observed for the survival in 60 mg $CuCl_2$ L^{-1}, where the nematodes survived on average longer than in 0 mg $CuCl_2$ L^{-1}. The differences between the populations originating from

different habitats were significant only in the highest concentration of copper tested. In this environment (180 mg $CuCl_2$ L^{-1}), the nematodes originating from plots of habitat S lived on average longer (Wilcoxon test, $P = 0.02$; log-rank test, $P = 0.058$) indicating greater resistance to copper.

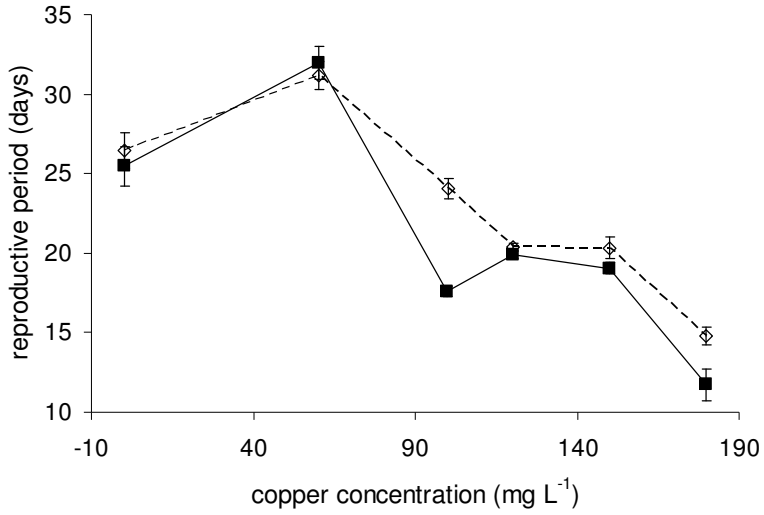


Figure 4: Response of reproductive period of A. nanus originating from the plots of habitat C (closed symbols, solid line, replicate populations pooled) and habitat S (open symbols, dashed line, replicate populations pooled) to different concentrations of copper in the agar medium. Mean ± SE is shown.

There was no significant difference between the populations in the response of juvenile period to copper. For both populations, juvenile period was on average longer in higher copper concentrations (log-rank test, $\chi^2 = 352$, $P < 0.001$). Higher tolerance to copper observed in populations from the habitat S, suggests that the nematodes became capable of dealing with higher copper concentrations. Since we used the fourth generation after introducing the nematodes to laboratory culture for this experiment, the observed differences are most likely genetically determined.

c) pH response experiment

Populations from the different field habitats showed divergent responses to pH in a number of life-history traits, namely, pH affected reproduction of populations of both origins ($F = 12.29$, $P < 0.001$). Neither population showed overall advantage in total reproduction across all pH levels ($F = 0.196$, $P = 0.65$). However, the nematodes from the populations originating from plots of habitat S laid on average more eggs on the medium of lower pH (4.0, 4.5) compared with the number of eggs laid on the medium of pH 5.0-6.0 (Fig 5a). At the same time, the nematodes originating from habitat C laid more eggs on the medium of higher pH (5.0-6.0) in comparison with the nematodes from habitat S. The significance of interaction term habitat × pH of medium ($F = 5.54$, $P < 0.001$) indicated differences in the pattern of pH response between populations from different habitats. The analysis of reproductive period

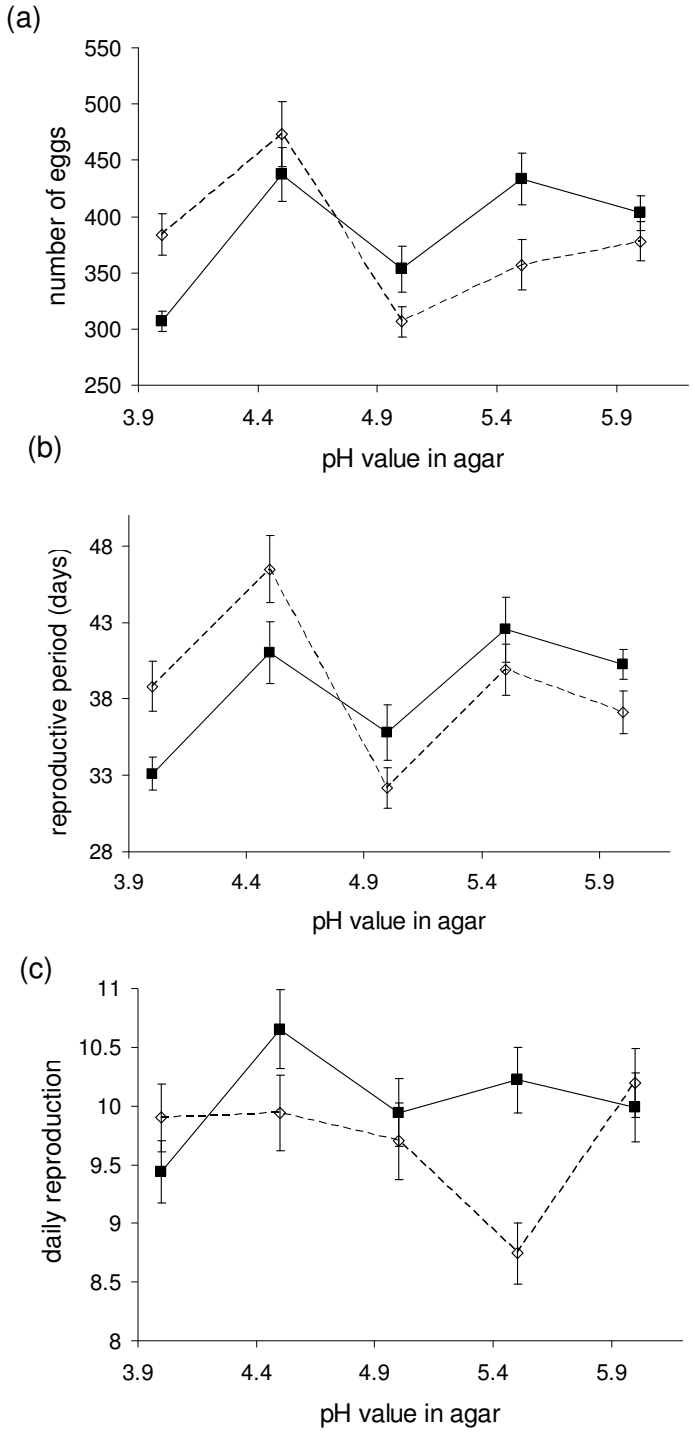


Figure 5: Reaction norms of the populations originating from habitat C (closed symbols, solid line, replicate populations pooled) and from habitat S (open symbols, dashed line, replicate populations pooled) to different levels of pH of the agar medium for (a) total reproduction, (b) reproductive period, (c) daily reproduction. Mean ± SE is shown.

revealed similar pattern of the response to pH like the one obtained for reproduction (Fig. 5b): the effect of pH was significant ($F = 11.063$, $P < 0.001$) and there was a significant effect of interaction habitat × pH of medium ($F = 4.653$, $P = 0.001$). Similar to total reproduction no habitat effect was detected ($F = 0.25$, $P = 0.62$). Daily reproduction was affected only by the interaction habitat × pH of medium ($F = 3.39$, $P = 0.01$) while the responses to pH and habitat were not significant ($F = 2.35$, $P = 0.054$ and $F = 3.45$, $P = 0.064$, respectively; Fig. 5c). The analysis of survival revealed substantial differences between the responses of the populations of different origin to pH (Fig. 6a, b). On agar medium with pH 4.0, the nematodes originating from habitat S survived longer (log-rank test, $\chi^2 = 9.397$, $P = 0.002$; Wilcoxon test, $\chi^2 = 7.148$, $P = 0.008$). Whereas, the situation reversed the medium of pH 6.0. In this case, the nematodes derived from habitat C survived longer (log-rank test, $\chi^2 = 2.302$, $P = 0.129$; Wilcoxon test, $\chi^2 = 8.390$, $P = 0.004$). Length of juvenile period was unaffected by pH (habitat S: log-rank test, $\chi^2 = 5.696$, $P = 0.223$; habitat C: log-rank test, $\chi^2 = 3.963$, $P = 0.411$). In addition, the origin of the populations had no influence on the response to pH (log-rank tests, $P > 0.05$ for all tests).

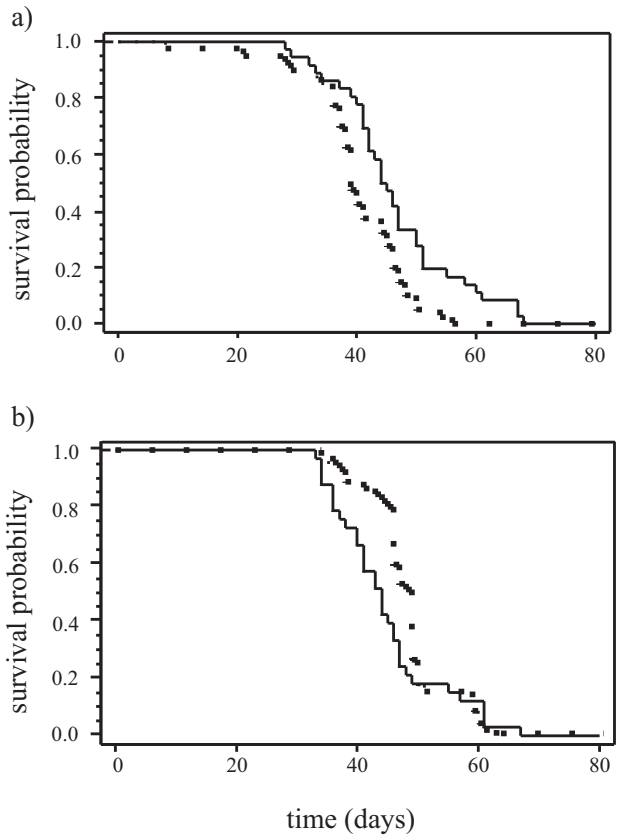


Figure 6: Survival probability of the nematodes originating from habitat C (dotted line, replicate populations pooled) and from habitat S (solid line, replicate populations pooled) on (a) pH 4.0 and (b) pH 6.0.

The results strongly suggest that the environment to which the nematodes were exposed in the field determined their response to pH in the laboratory in a number of life-history traits. Since the populations displayed higher values of the traits like survival and total reproduction in the pH conditions that corresponded to the field conditions they evolved in for 20 years, it can be concluded that the differences between these values represent adaptive changes as response to selective pressure that was imposed by pH level in the field.

Discussion

While asexual reproduction is supposed to assure the maintenance of a superior genotype in a stable environment, sexual reproduction is considered to provide the basis for rapid evolution. Our results indicated rapid adaptive divergence within the asexual population of *A. nanus* in response to locally imposed selection. Selective agents and time-scale were defined by application of an evolution experiment with manipulated environmental variables in a randomized block design. To our knowledge, this kind of approach has not been used in the studies of adaptive divergence of natural populations until today.

The evidence collected over the last four decades indicated a considerable intraclonal variation in many asexually reproducing species (reviewed in Loxdale & Lushai 2003; Lushai *et al.* 2003). Moreover, artificial selection studies under laboratory conditions showed that populations reproducing by obligate parthenogenesis were able to respond rapidly to strong selection. For example Wilhoit and Mittler (1991) demonstrated phenotypic response to selection on the body size within 14-16 generations in the greenbug *Schizaphis graminum*. In another study, divergent selection on weight-specific fecundity (WSF) imposed on asexual lineages of *Daphnia pulex* resulted in the differentiation of WSF within two generations (Gorokhova *et al.* 2002). The most rapid response that could be attributed to selection was reported for the pea aphid, *Acyrthosiphon pisum*, selected for different predator defence behaviour. The differences resulting from the disruptive selection were apparent after a single generation (Andrade & Roitberg 1995). However, the evidence for rapid adaptive response for asexual populations in the field is poor. The rare exceptions include the case study of the spotted alfalfa aphid, *Therioaphis maculata*, where a small introduced asexual population has evolved into numerous variants within a few generations (Dickson 1962).

Although asexual reproduction is expected to maintain local adaptation, only a few cases of adaptive divergence within asexual natural populations have been reported. Prati and Schmid (2000) and Lenssen *et al.* (2004) showed that the clonal plant *Ranunculus reptans* locally adapted to flooding regime. Another group of studies

showed adaptation of various asexual species to their host. Such adaptation was shown for *Sitobion* aphids (Sunnucks *et al.* 1998), the spider mite *Brevipalus phoenicis* (Groot *et al.* 2005) and the wheat yellow rust *Puccinia striiformis* f. sp. *tritici* (Enjalbert *et al.* 2005). Some molecular studies on aphid species provided evidence for local selection (reviewed in Lushai *et al.* 2003).

Testing local adaptation requires the incorporation of reaction norm or reciprocal transplant experiments (Kawecki & Ebert 2004). In order to interpret the observed life-history patterns as resulting from adaptation, in both reciprocal transplant and reaction norm experiments, the assumption of positive correlation of the analysed trait with fitness must be fulfilled. Our reaction norm experiment demonstrated clear differences between populations of both habitats in a number of fitness related traits. The traits analyzed in reaction norm experiments, like total reproduction and survival, influence fitness directly and therefore deliver a straightforward interpretation of the results. In the reciprocal transplant experiment we analysed the MRGR of the nematodes. The relationship of body growth rate with fitness is expected to be less direct than the one with reproduction or survival. Nevertheless, the results of the transplant experiment are consistent with the findings of the reaction norm experiments.

In the reciprocal transplant experiment, we used defaunated soil from the experimental field from where the nematodes originated. Since, in the field, nematodes are likely to experience negative interactions with competing, predacious and pathogenic species, use of defaunated soil could result in releasing the pressure caused by these interactions. As a consequence, MRGR values measured in the experiment are expected to be, on average, higher than in the field. Additionally, since the strength of interspecific interactions can differ between stressed and unstressed habitats (Morin 1999), we cannot exclude the possibility that the pattern of MRGR response in defaunated soil does not entirely overlap with the MRGR response under field conditions. Regardless of these limitations, the results of the implemented transplant experiment can be regarded as an indication for adaptation to local abiotic soil conditions as the observed response could be attributed to the differences in soil properties defined by the experimental manipulation (copper load and soil pH).

There are several aspects that should be taken into consideration while interpreting local adaptation events. Dispersal rate is one of them, since it has profound influence on the capability of local populations to diverge. It is known that individuals of *A. nanus* can passively disperse by wind (Lee 2002) and through human activities, such as ploughing. The active dispersal in soil is not likely to play a major role in gene flow of nematodes, since the distance travelled per year for most examined nematode species is estimated to range from several centimetres to several meters (Hunt *et al.* 2001). In general, the migration of nematodes among the experimental plots can be regarded as limited. The second factor that is relevant for

the adaptive potential of the populations under selection is mutation rate. There are no estimates of genome-wide mutation rate for *A. nanus*, but the recent studies on the spontaneous mutation rate in inbred lines of *Caenorhabditis elegans* using DNA sequencing imply that the mutation rate is high, approximately ~2.1 mutations per genome per generation (Denver *et al.* 2004). Based on the comparative analysis of SSU rDNA between different nematode clades it can be concluded that the mutation rate of *A. nanus* is likely to be of comparable magnitude as the one of *C. elegans* (A.W.G. van der Wurff, unpublished data). Such high mutation rate would facilitate adaptation by increasing genetic variation (De Visser & Rozen 2005). Other factors that operate together with mutation rate are population size and generation time. We found that the populations inhabiting a single plot (6 × 11 m), regardless of habitat type, consisted of approximately $2 \times 10^5 – 5 \times 10^5$ individuals. This is a relatively low number compared to those of prokaryotic organisms or of some eukaryotic species that were found to respond fast with adaptation to a change in environment (Lushai *et al.* 2003). In addition, generation time of *A. nanus* under field conditions is relatively long (number of generations per year was estimated as 0.7 and 6.6 for the nematodes from habitat S and habitat C, respectively). Such long generation times together with relatively low population size suggests that high mutation rate could be considered the main factor to facilitate rapid adaptive divergence within the population of *A. nanus*.

Since the level of genetic variation in parthenogenetic species does not depend on mutation rate alone, the knowledge about other genetic mechanisms that operate in *A. nanus* would contribute to a better understanding of its adaptation. Polyploidy, which is common in mitotic parthenogenetic nematodes, is expected to result in higher levels of genetic variation through increased levels of heterozygosity (Lokki 1976). Additionally, certain aspects of chromosome nature and egg development have the potential to result in higher levels of genetic variation in parthenogenetic species (Asher 1970). The studies on egg development in *A. nanus* indicated maturation division equivalent to first meiotic division in sexual species, which results in the formation of a single large polar body. Therefore, parthenogenesis in *A. nanus* can be regarded as essentially mitotic. In rare cases, a second meiotic division was observed with subsequent fusion of oocyte nucleus and second polar body which was concluded to restore diploidy (Lahl *et al.* 2006). These observations suggest that neither higher ploidy level nor the fusion of the second polar body with the oocyte nucleus is likely to play significant role in generating higher heterozygosity levels and, consequently, higher genetic variation. On the other hand, in the taxon Nematoda (with few exceptions) chromosomes have a holokinetic nature (Maddox *et al.* 2004), which is expected to increase the frequency of chromosomal rearrangements. Overall, because mechanisms responsible for high adaptability of this species remain unresolved, there is a need for analysis of the levels and sources of genetic variation in populations of *A. nanus*. Genetic analysis could also help to answer the question about

cryptic sex. Since cryptic sex can influence adaptive potential of species that reproduce predominantly by parthenogenesis; discovering such events in *A. nanus* would require revision of the conclusions of the presented work.

The copper response experiment indicated higher survival and reproduction of the nematodes from habitat S in higher copper concentrations. These results suggest that some kind of detoxification or repair mechanisms (probably costly in terms of energy) may be involved. It has been demonstrated that some metal-tolerant populations exhibit lower metabolic rate and slow growth to conserve energy or other resources (Antonovics *et al.* 1971; Donker 1992). Contrary to the results of these studies, our results suggest that the nematodes from habitat S could be even selected for an increase in metabolic rate. This could be concluded from earlier onset of reproduction of the nematodes from habitat S in the control test medium (Cu = 0) and their higher daily reproductive output in higher copper concentrations (data not shown). The results of the pH response experiment also support this scenario. The nematodes performed on an average better in the pH levels corresponding to the pH of the environment from where they originated. This type of response was observed not only for survival but also for total reproduction and reproductive period, which suggests that the improvement in survival was not coupled with the decrease in metabolic rate.

In conclusion, the present evidence for rapid local adaptation provides strong support for high adaptive potential of *A. nanus*. The evolution of specialized phenotypes in this species indicates that local adaptation may actually contribute more to the determination of population structure and distribution of asexual species than previously acknowledged. Studies of phenotypic evolution in asexual populations in replicated evolution experiments provide the opportunity to test hypotheses concerning the direction and the magnitude of phenotypic changes in the context of known selective agent, time- and geographical scale. Combination of this approach with population genetic analysis of population structure in future studies would provide a powerful tool to reveal mechanisms behind evolutionary dynamics of asexual populations.

Acknowledgements

We thank André van der Wurff for his comments on the manuscript, Sven Boström and Tom Bongers for their information about reproduction in *Acrobeloides nanus*. We also thank Ervin Temminghoff for the use of the field sites. This research was funded by the TRIAS Programme (TRIpartite Approach to Soil system processes) of Delft Cluster, SKB (Foundation for development and transmission of soil knowledge) and NWO (the Netherlands Organisation for Scientific Research), as project 835.80.008.

References

Andrade, M.C.B. & Roitberg, B.D. (1995) Rapid response to intraclonal selection in the pea aphid (*Acyrthosiphon pisum*). *Evolutionary Ecology*, **9**, 397-410.

Antonovics, J., Bradsham, A.D. & Turner, R.G. (1971) Heavy metal tolerance in plants. *Advances in Ecological Research*, **7**, 1–85.

Aronson, J., Kigel, J., Shmida, A. & Klein, J. (1992) Adaptive phenology of desert and mediterranean populations of annual plants grown with and without water-stress. *Oecologia*, **89**, 17-26.

Arts, M.S.J., Schill, R.O., Knigge, T., Eckwert, H., Kammenga, J.E. & Kohler, H. (2004) Stress proteins (hsp70, hsp60) induced in isopods and nematodes by field exposure to metals in a gradient near Avonmouth, UK. *Ecotoxicology*, **13**, 739-756.

Asher, J.H. (1970) Parthenogenesis and genetic variability.2. One-locus models for various diploid populations. *Genetics*, **66**, 369-&.

Berglund, A.B.N., Dahlgren, S. & Westerbergh, A. (2004) Evidence for parallel evolution and site-specific selection of serpentine tolerance in *Cerastium alpinum* during the colonization of Scandinavia. *New Phytologist*, **161**, 199-209.

Bijlsma, R. & Loeschcke, V. (2005) Environmental stress, adaptation and evolution: an overview. *Journal of Evolutionary Biology*, **18**, 744-749.

Bird, A.F. (1971) Specialized adaptations of nematodes to parasitism. *Plant Parasitic Nematodes* (eds B.M. Zuckerman, W.F. Mai & R.A. Rohde), pp. 35-49. Academic Press, New York.

Bird, A.F., De Ley, P. & Bird, J. (1993) Morphology, oviposition and embryogenesis in an Australian population of *Acrobeloides nanus*. *Journal of Nematology*, **25**, 607-615.

Bongers, T. (1988) *De nematoden van Nederland* Koninklijke Nederlandse Natuurhistorische Vereniging, Utrecht.

Campbell, B.D. & Grime, J.P. (1992) An experimental test of plant strategy theory. *Ecology*, **73**, 15-29.

De Goede, R.G.M. & Bongers, T. (1998) *Nematode communities of northern temperate grassland ecosystems* Focus, Giessen.

De Visser, J.A.G.M. & Rozen, D.E. (2005) Limits to adaptation in asexual populations. *Journal of Evolutionary Biology*, **18**, 779-788.

Denver, D.R., Morris, K., Lynch, M. & Thomas, W.K. (2004) High mutation rate and predominance of insertions in the *Caenorhabditis elegans* nuclear genome. *Nature*, **430**, 679-682.

Dickson, R.C. (1962) Development of the spotted alfalfa aphid population in North America. *Internationaler Kongress fur Entomologie (wien 1960)*, **2**, 26-28.

Donker, M.H. (1992) Energy reserves and distribution of metals in populations of the isopod *Porcellio scaber* from metal-contaminated sites. *Functional Ecology*, **6**, 445-454.

Emmons, S.W., Klass, M.R. & Hirsh, D. (1979) Analysis of the constancy of DNA sequences during development and evolution of the nematode *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences, USA*, **76**, 1333-1337.

Enjalbert, J., Duan, X., Leconte, M., Hovmoller, M.S. & De Vallavieille-Pope, C. (2005) Genetic evidence of local adaptation of wheat yellow rust (*Puccinia striiformis f. sp tritici*) within France. *Molecular Ecology*, **14**, 2065-2073.

Freckman, D.W. (1982) Nematode contributions to ecosystem. *Nematodes in Soil Ecosystems* (ed D.W. Freckman), pp. 81-97. University of Texas Press, Austin.

Futuyma, D.J. & Moreno, G. (1988) The evolution of ecological specialization. *Annual Review of Ecology and Systematics*, **19**, 207-233.

Goddard, M.R., Godfray, H.C.J. & Burt, A. (2005) Sex increases the efficacy of natural selection in experimental yeast populations. *Nature*, **434**, 636-640.

Goldstein, B., Frisse, L.M. & Thomas, W.K. (1998) Embryonic axis specification in nematodes: evolution of the first step in development. *Current Biology*, **8**, 157-160.

Gorokhova, E., Dowling, T.E., Weider, L.J., Crease, T.J. & Elser, J.J. (2002) Functional and ecological significance of rDNA intergenic spacer variation in a clonal organism under divergent selection for production rate. *Proceedings of the Royal Society B: Biological Sciences*, **268**, 2373-2379.

Grime, J.P. & Hunt, R. (1975) Relative growth-rate - its range and adaptive significance in a local flora. *Journal of Ecology*, **63**, 393-422.

Groot, T.V.M., Janssen, A., Pallini, A. & Breeuwer, J.A.J. (2005) Adaptation in the asexual false spider mite *Brevipalpus phoenicis*: evidence for frozen niche variation. *Experimental and Applied Acarology*, **36**, 165-176.

Hunt, H.W., Wall, D.H., De Crappeo, N.M. & Brenner, J.S. (2001) A model for nematode locomotion in soil. *Nematology*, **3**, 705-716.

Kawecki, T.J. & Ebert, D. (2004) Conceptual issues in local adaptation. *Ecology Letters*, **7**, 1225-1241.

Korthals, G.W., Alexiev, A.D., Lexmond, T.M., Kammenga, J.E. & Bongers, A.M.T. (1996) Long-term effects of copper and pH on the nematode community in an agroecosystem. *Environmental Toxicology and Chemistry*, **15**, 979-985.

Korthals, G.W. (1997) *Pollutant induced changes in terrestrial nematode communities* Wageningen Agricultural University, Wageningen.

Lahl, V., Sadler, B. & Schierenberg, E. (2006) Egg development in parthenogenetic nematodes: variations in meiosis and axis formation. *International Journal of Developmental Biology*, **50**, 393-397.

Laugsch, M. & Schierenberg, E. (2004) Differences in maternal supply and early development of closely related nematode species. *International Journal of Developmental Biology*, **48**, 655-662.

Lee, D.L., ed. (2002) *The biology of nematodes* Taylor & Francis, London.

Lenssen, J.P.M., Van Kleunen, M., Fischer, M. & De Kroon, H. (2004) Local adaptation of the clonal plant *Ranunculus reptans* to flooding along a small-scale gradient. *Journal of Ecology*, **92**, 696-706.

Lenz, R. & Eisenbeis, G. (1998) An extraction method for nematodes in decomposition studies using the minicontainer-method. *Plant and Soil*, **198**, 109-116.

Lokki, J. (1976) Genetic polymorphism and evolution in parthenogenetic animals .8. Heterozygosity in relation to polyploidy. *Hereditas*, **83**, 65-72.

Loxdale, H.D. & Lushai, G. (2003) Rapid changes in clonal lines: the death of a 'sacred cow'. *Biological Journal of the Linnean Society*, **79**, 3-16.

Lushai, G., Loxdale, H.D. & Allen, J.A. (2003) The dynamic clonal genome and its adaptive potential. *Biological Journal of the Linnean Society*, **79**, 193-208.

Lynch, M. (1984) Destabilizing hybridization, general-purpose genotypes and geographic parthenogenesis. *Quarterly Review of Biology*, **59**, 257-290.

Maddox, P.S., Oegema, K., Desai, A. & Cheeseman, I.M. (2004) "Holo"er than thou: Chromosome segregation and kinetochore function in *C. elegans*. *Chromosome Research*, **12**, 641-653.

McNeilly, T. (1968) Evolution in closely adjacent plant populations III. *Agrostis tenuis* on a small copper mine. *Heredity*, **23**, 99-108.

Morin, P.J. (1999) *Community ecology* Blackwell Science, New Brunswick, New Jersey.

Nesterov, P.I. (1979) *Plant-parasitic and free-living nematodes in Southwestern SSSR* USSR 'Shtiintsa', Kishinev.

Oostenbrink, M. (1960) Estimating nematode populations by some selected methods. *Nematology* (eds J.N. Sasser & W.R. Jenkins), pp. 85-102. The University of North Carolina Press, Chapel Hill, NC.

Posthuma, L. & Van Straalen, N.M. (1993) Heavy-metal adaptation in terrestrial invertebrates - a review of occurrence, genetics, physiology and ecological consequences. *Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology*, **106**, 11-38.

Prati, D. & Schmid, B. (2000) Genetic differentiation of life-history traits within populations of the clonal plant *Ranunculus reptans*. *Oikos*, **90**, 442-456.

Radford, P.J. (1967) Growth analysis formulae - their use and abuse. *Crop Science*, **7**, 171-175.

Rice, K.J. & Mack, R.N. (1991) Ecological genetics of *Bromus tectorum* .3. The demography of reciprocally sown populations. *Oecologia*, **88**, 91-101.

SAS Institute (2000). SAS Statistical Software. In. NC: SAS Institute Inc, Cary.

Sohlenius, B. & Boström, S. (1999) Effects of global warming on nematode diversity in a Swedish tundra soil - a soil transplantation experiment. *Nematology*, 695-709.

Stanton, M.L., Roy, B.A. & Thiede, D.A. (2000) Evolution in stressful environments. I. Phenotypic variability, phenotypic selection, and response to selection in five distinct environmental stresses. *Evolution*, **54**, 93-111.

Sunnucks, P., Chisholm, D., Turak, E. & Hales, D.F. (1998) Evolution of an ecological trait in parthenogenetic *Sitobion* aphids. *Heredity*, **81**, 638-647.

Travisano, M. & Lenski, R.E. (1996) Long-term experimental evolution in Escherichia coli .4. Targets of selection and the specificity of adaptation. *Genetics*, **143**, 15-26.

Triantaphyllou, A.C. (1973) Environmental sex differentation of nematodes in relation to pest management. *Annual Review of Phytopathology*, **11**, 441-462.

Van Bezooijen, J. (1997) *Methoden en technieken voor nematologie*, Wageningen University and Research Centre, Wageningen.

Wiegner, O. & Schierenberg, E. (1998) Specification of gut cell fate differs significantly between the nematodes *Acrobeloides nanus* and *Caenorhabditis elegans*. *Developmental Biology*, **204**, 3-14.

Wilhoit, L.R. & Mittler, T.E. (1991) Biotypes and clonal variation in greenbug (*Homoptera, Aphididae*) populations from a locality in California. *Environmental Entomology*, **20**, 757-767.

Wood, W.B. (1988) *The nematode Caenorhabditis elegans* Cold Spring Harbor Laboratory Press, New York.

Chapter 4

Rapid divergence of genetic variance-covariance matrix within a natural population

Agnieszka Doroszuk
Marcin W. Wojewodzic
Gerrit Gort
Jan E. Kammenga

Submitted to *The American Naturalist*

Abstract

The matrix of genetic variances and covariances (**G** matrix) represents genetic architecture of multiple traits sharing developmental and genetic processes and is central for predicting phenotypic evolution. These predictions require that **G** matrix is stable. Yet, the time-scale and conditions promoting **G** matrix stability in natural populations remain unclear. Here, we studied the stability of **G** matrix in a 20 year-long evolution field experiment in a population of the cosmopolitan parthenogenetic soil nematode *Acrobeloides nanus*. The population was subjected to drift and divergent selection (control and stress conditions) which led to adaptive divergence of life-history traits. Selection regime did not influence the level of absolute genetic constraints: under both regimes two genetic dimensions for six life-history traits were identified. A strong response to selection was found for other aspects of **G** matrix structure, namely, the matrices of both selection regimes shared only one common principal component. **G** structure was also influenced by drift, with higher divergence under control conditions. These results show that **G** matrix might evolve rapidly in natural populations. The observed high dynamics of **G** structure is likely to represent the general feature of asexual species and limits the predictive power of **G** in phenotypic evolution analyses.

Introduction

Evolutionary change in a quantitative trait is commonly accompanied by simultaneous changes in other traits through shared developmental, functional and genetic processes. **G** matrix summarizes these relationships and can, therefore, be used to make predictions of future response to selection of correlated traits or to obtain the estimation of the selection pressure acting in the past (Lande 1979, 1980; Cheetham *et al.* 1994; Bjorklund 1996; Phillips & McGuigan 2006). Evolutionary change in multiple quantitative traits can be described by the multivariate extension of the breeder's equation

$$\Delta\bar{z} = \mathbf{G}\mathbf{P}^{-1}\mathbf{S} \qquad \text{eqn 1}$$

where $\Delta\bar{z}$ is the vector of population mean responses, **G** is the matrix of additive genetic variances and covariances, **P** is the matrix of phenotypic variances and covariances and **S** is the vector of selection differentials (Lande 1979; Lande & Arnold 1983). This equation is valid only under the assumption that **G** is stable over evolutionary time. Theoretical and empirical investigations show, however, that stability of **G** cannot be ensured (Steppan *et al.* 2002; McGuigan 2006). Especially over longer evolutionary times **G** matrices are likely to diverge, which was demonstrated by several studies comparing **G** matrices among natural populations of different taxa (Paulsen 1996; Roff & Mousseau 1999). Additionally, experimental laboratory studies provided the evidence that strong selection or drift may lead to rapid changes in **G** structure (but see Wilkinson *et al.* 1990; Shaw *et al.* 1995; Phillips *et al.* 2001).

Since the problem of **G** stability seems not to have a definite solution, it should be considered from the perspective of specific conditions, which might promote **G** stability or act in a destabilizing way (Turelli 1988; Jones *et al.* 2003). The influence of these conditions (selection, drift) can be best understood when the changes in **G** matrices are interpreted in relation to their known operation time. One of the factors affecting **G** stability is recombination frequency and, related to it, mode of reproduction (Phillips & McGuigan 2006). In principle, genomes of asexual species are transmitted to new generations without recombination. As a result, selection acts upon the properties of the genome as a whole. In such populations, genetic means, variances and covariances are influenced by non-additive genetic effects and by genetic disequilibria to a greater extent than in the corresponding sexual populations (Kelly 1999; Pfrender & Lynch 2000). Genetic disequilibria are expected to accumulate over the period of asexual reproduction leading to destabilization of **G** structure. While in cyclical parthenogens the changes in **G** matrices acquired during the periods of asexual reproduction are transient (Deng & Lynch 1996; Pfrender & Lynch 2000), in obligate parthenogens the changes persist and, as a consequence, their genetic architecture is likely to be unstable. The prediction of limited stability of **G** matrices in asexual

species has never been tested in natural populations. In addition, the patterns and dynamics of the changes in **G** structure in asexuals under selection and drift remain unexplored.

Many processes (i.e. mutation, migration, selection and drift) are expected to influence the **G** structure in different ways. Dimensionality of **G** is the most general aspect of **G** structure and is related to the number of genetically independent traits represented by a set of phenotypic traits which make up the 'phenotypic space'. When breeding values are concerned the genetic traits may fall in the subspace of this phenotypic space meaning that there are absolute constraints on evolution (Kirkpatrick & Lofsvold 1992; Mezey & Houle 2005). Concerning other aspects of **G** structure, it has been suggested that random drift induces proportional changes of **G**, while selection is expected to cause non-proportional structural changes (Roff *et al.* 1999; Roff 2000). Although these relationships are sometimes used to infer which processes contributed to the observed differences between populations' **G** matrices (e.g. Roff *et al.* 1999; Marroig & Cheverud 2004), their reliability as a conclusive criterion has been questioned. Namely, it has been argued that proportional changes are rather an average response to drift and individual populations may display a broader range of divergence levels (Phillips *et al.* 2001).

As theoretical approaches often struggle with a high level of complication and unrealistic assumptions (Jones *et al.* 2004), empirical studies are valuable means to investigate stability of **G** matrices (McGuigan 2006). The empirical studies can be classified into two main groups: comparative studies of natural populations and laboratory evolution studies. The advantage of comparative analyses is that they reveal direction of the changes in **G** that actually happen in nature. On the other hand, they often suffer from the lack of information on populations' phylogenies and history. It complicates relating the observed pattern of **G** structure to the time-scale of population differentiation (Phillips & McGuigan 2006). In addition, the knowledge of the nature of selection is usually limited. Laboratory studies provide a framework for testing the role of evolutionary processes in shaping **G** structure, however, under unrealistic laboratory conditions.

Natural experiments or planned experiments involving natural populations in the field combine the advantages of both approaches. Namely, they provide a setting to make testable predictions without a need to compromise the realism of field conditions.

We investigated the stability of **G** in response to imposed selection and drift in natural populations of a parthenogenetic, soil-dwelling nematode *Acrobeloides nanus* de Man 1880 (Nematoda, Cephalobidae). *A. nanus* can be found in soils of various physical and chemical properties (Bird *et al.* 1993; Korthals *et al.* 1996; De Goede & Bongers 1998) and constitutes a large part of soil nematode communities across extremely different habitats like deserts of Australia (Bird *et al.* 1993) and Swedish

tundra (Sohlenius & Boström 1999). The analysed populations originated from an experimental field, where combined treatments of two stress factors (pH level and copper) were applied approximately 20 years ago in a replicated and randomized block design and maintained thereafter. In that way, the population of *A. nanus*, which inhabited the field before the treatment application, was divided into a number of subpopulations exposed to different treatments of the stress factors. We analysed subpopulations from two extreme treatments (control treatment, habitat C, and stress treatment, habitat S; 4 replicate subpopulations per treatment) and demonstrated adaptive divergence of life-history traits in response to control and stress conditions (chapter 3, this thesis).

Here we characterise **G** matrices of life-history traits of the same subpopulations using common garden laboratory experiment and two methods of matrix comparison: factor-analytic approach (Hine & Blows 2006) and Flury hierarchy (Phillips & Arnold 1999). We attempt to determine the effects of selection on **G** structure (i.e. dimensionality, proportionality, common principal components). The insight into the effects of drift is obtained by the comparison of **G** matrices among replicate populations within selection treatments. Additionally, we evaluate the effects of these evolutionary forces on phenotypic (**P**) and environmental (**E**) matrices.

Materials and methods

Study species, experimental field and sampling

A. nanus is a free-living, bacterial-feeding nematode that reproduces by parthenogenesis (Wiegner & Schierenberg 1998; Laugsch & Schierenberg 2004). Under laboratory conditions, at 20 °C, the juvenile period is approximately 10 days and 250 eggs are laid over the period of 35 days. The post-reproductive period is nearly 10 days.

The experimental field at Bovenbuurt is located ca. 3 km NNE of Wageningen, the Netherlands (Korthals *et al.* 1996). It was created in 1982, when an agricultural field was divided into 128 plots of 6 × 11 m each and four copper and pH levels were introduced by applying once $CuSO_4 \cdot 5H_2O$ and sulphur powder or ground calcitic limestone, respectively in a full factorial design. pH was readjusted every five years. The plots were arranged into eight blocks, each with a random distribution of all combinations of copper and pH treatments. Only two extreme treatments were used (each represented by four replicate plots): one treatment imposing stress by low pH (4.0) and the copper concentration of 750 kg Cu ha^{-1} (habitat S) and a control treatment with pH 6.1 without copper addition (habitat C). Maize, potatoes and oats have been grown since 1983 in a three-year crop rotation regime. More information

about *A. nanus* and the experimental field can be found in chapters 2 and 3 in this thesis.

About 30 cores (diameter 30mm) from the top 10 cm of soil mineral layer were collected from each plot and mixed to obtain soil samples representative for the plots. Nematodes were isolated with standard techniques (Oostenbrink 1960) and the individuals identified as *A. nanus* (microscope at 400× total magnification) were placed on Petri dishes (one individual per dish) with proteose peptone agar medium (PPA; 2% technical agar, 0.5% proteose peptone) and *Acinetobacter johnsonii* strain 210A (the Netherlands Culture Collection of Microorganisms, access number LMAU A130) (Bonting *et al.* 1992), a soil bacterium, as a food source. In this way approximately 15 laboratory populations per plot were established. Number of generations per year under field conditions was estimated as 0.7 for the nematodes from habitat S and 6.6 for the nematodes form habitat C (chapter 2, this thesis).

Common garden experiment

Individual life-history traits of individuals were determined with the use of 24-well dishes. The PPA medium (200 μl droplets, pH 6.0) was pipetted on the inner side of the lid while wells contained 0.5 mL of water to prevent the medium from drying out. On the top of every agar droplet, 2 μL of *A. johnsonii* suspension ($2 \cdot 10^8$ cells mL^{-1}) was applied and the dishes were subsequently incubated at 28 °C overnight.

We define all nematodes derived from a single individual isolated from an experimental plot as a clone. We used 8-15 clones per experimental plot (of both habitats). Six synchronized eggs per clone were placed in separate wells and subsequently the individuals that hatched from these eggs were transferred every second day to new wells. The used eggs were the fourth generation after introduction of the nematodes to the laboratory. On the basis of daily observation we determined for each individual the duration of reproductive period (rp), total number of offspring (tr), daily reproduction (dr) and life span (ls). Additionally, in order to analyse the reproductive output in different stages of reproductive period, number of offspring during first 12 days of reproduction (e12) and between 13th and 24th day (e12a) were assessed. The multiwell dishes with nematodes were incubated at 20 °C in dark.

Statistical methods

All traits were standardized to the mean of 0 and standard deviation of 1. The basis of genetic analysis was the use of the 8-15 clones originating from each experimental plot. Partitioning of total phenotypic variance into the within- and among-clone variance allowed estimating total genetic variance. Among-clone variance reflected total genetic variance (additive and non-additive) together with the maternal effects

(Lynch & Walsh 1998). Although we did not assess the magnitude of maternal effects, the influence of maternal effect on the results of the experiment was equalized by rearing the former generation of nematodes in standardized, similar laboratory conditions. Broad-sense heritability (H^2) was estimated as the proportion of total variance due to among-clone variance. The quantitative genetic parameters and phenotypic covariances as presented in the Results section were estimated with *h2boot* software available at www.uoregon.edu/~pphil/programs/.

Comparison of the matrices

a) Factor-analytic approach

The factor-analytic modelling of a covariance matrix aims at finding the minimum number of factors or dimensions explaining the covariances among a number of traits. It can be used for direct estimation of genetic principal components (Kirkpatrick & Meyer 2004; Hine & Blows 2006). The estimation procedure is based on restricted maximum-likelihood (REML), allowing likelihood ratio testing of the number of genetic dimensions explaining genetic covariation among traits. It also allows the comparison of the structure of multiple **G** matrices for any experimental design. Hine and Blows (2006) outlined the readily available factor-analytic approach implemented in the PROC MIXED in SAS (SAS Institute). For the purpose of our study the hypotheses testing was divided into two steps. In the first step, **G** dimensionality was tested for each habitat separately. The reduced-rank covariance model for trait was specified for the clone level using the FA0(q) covariance structure (q is the number of dimensions of **G**) of PROC MIXED, assuming independence between the clones. An unstructured covariance matrix was assumed for trait at the level of individuals. The used linear mixed model specified trait, plot and their interaction as fixed factors, removing in that way differences in means between the trait-plot combinations. Six nested hypothesis tests were performed starting from the full model where the number of factors (dimensions) was equal to the number of traits and continued by dropping the factors sequentially. When the dimensionality of **G** for both habitats was determined with log-likelihood ratio tests, the second step of the analysis was performed to assess the effect of the habitat on the reduced-rank **G** matrices. The fixed part of the mixed model contained plot, trait and their interactions, eliminating mean differences between the combinations. The same random part of the mixed model was specified as in the analysis per habitat, but now allowing for differences in FA0(q) structures between the two habitats (using the 'Group' option of PROC MIXED). This model was compared with the model with an equal FA0(q) structure for the two habitats, using a likelihood ratio test.

b) Flury hierarchy

The Flury hierarchy method is a principal components method that allows comparison of two or more matrices along the hierarchy of hypotheses relating to the level of their similarity (Phillips & Arnold 1999). Compared matrices can have unrelated structure or can share some principal components (eigenvectors). Further, matrices can share all eigenvectors while their eigenvalues might differ by a single constant indicating proportionality. Finally, matrices can be equal having the same values at each element. The fit of each model to the observed matrices is determined by the log-likelihood statistics. The hypotheses are tested with a likelihood ratio against the model of unrelated structure. The testing starts at the bottom (where the first principal component is tested) through all levels towards matrix equality. When a significant deviation from the unrelated structure is encountered, the testing is terminated (jump up procedure, Phillips & Arnold 1999). In this study, the significance of each test was determined with the use of randomization procedure (10000 runs per test), where clones were randomly assigned to different habitats (or plots in case of the within-habitat comparisons) and the matrices were tested for similarity. The obtained distribution of the test statistics was used as the null distribution for test at all hierarchy levels. This analysis was performed using the *CPCrand* software, which is available at www.uoregon.edu/~pphil/programs/.

Since this approach does not allow more complicated experimental designs, we performed three separate analyses. In the first analysis, the data for all plots within habitats were pooled, in order to investigate the matrix similarity between the habitats. The other two analyses were performed for each habitat separately to investigate the similarity of the **G** matrices among the replicate plots within habitat. Since we encountered the problem of non-positive matrices in our analyses, it was necessary to apply 'bending' procedure. This procedure adjusts non-positive eigenvalues it the way that the matrix becomes positive definite.

Results

Phenotypic means

Multivariate analysis of variance (MANOVA) with habitat as a fixed factor and plot (replicate population) nested within habitat showed a significant overall effect of habitat on phenotypic means of *A. nanus* life-history traits (Wilks' $\lambda = 0.80$, $F_{6, 340} = 13.84$, $P < 0.0001$) as well as a significant effect of plot (Wilks' $\lambda = 0.73$, $F_{36, 496} = 3.10$, $P < 0.0001$). The most pronounced differences between habitats were observed in daily reproduction and number of offspring during the first 12 days of reproduction. For both of these reproductive traits, the nematodes from the plots of habitat C showed lower values (Fig. 1). The differences between the habitats in the

trait means together with the results of the previous study of life-history traits implementing reaction-norm and transplant experiments in the same populations (chapter 3, this thesis) provide a strong support for an adaptive response to the imposed selection. Such response is likely to be accompanied by the changes in genetic covariance structure of the analyzed traits.

Heritability and genetic correlations

The analysis of broad-sense heritability (H^2), for the data pooled within habitats, revealed all six life-history parameters displayed significant levels of expressed genetic variation in the populations from habitat S. For the populations from habitat C, heritability values of life-span and length of reproductive period were not significant (Table 1). When averaged over all traits within habitat, the estimated heritability (SE values are given in the parentheses) for habitat C was 0.28 (0.074) and for habitat S H^2 was estimated as 0.20 (0.07). The analysis of genetic correlations indicated six significant correlations out of 15 for habitat C and nine significant correlations for habitat S (Table 1).

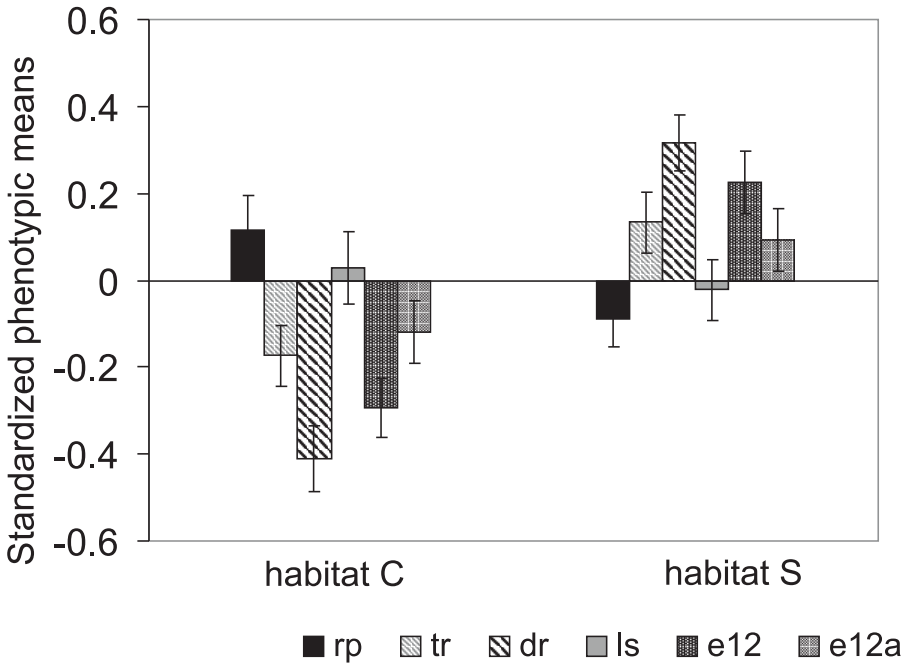


Figure 1: *Phenotypic means of life-history traits of A. nanus from different habitats standardized to general mean = 0 and SD = 1 (data within habitat pooled). Bars represent SE. The abbreviations used for the life-history traits are described in the text of Materials and Methods section.*

Table 1: Genetic correlations, covariances and broad-sense heritability for life-history traits of the populations of A. nanus from different habitats (data within habitats pooled).

	tr	dr	ls	rp	e12	e12a	H^2
tr	–	0.82 (0.14)***	-0.24 (1.16)	0.44 (0.73)	0.85 (0.14)***	1.00 (0.18)***	0.28 (0.10)***
	–	0.54 (0.24)*	0.92 (0.91)*	0.82 (0.25)*	0.77 (0.14)**	0.92 (0.15)**	0.20 (0.07)**
dr	0.26 (0.07)***	–	0.24 (1.45)	-0.07 (0.52)	0.97 (0.04)***	0.78 (0.40)**	0.54 (0.06)***
	0.11 (0.05)*	–	0.14 (0.65)	-0.01 (0.30)	1.00 (0.07) ***	0.20 (0.30)	0.20 (0.06)***
ls	-0.04 (0.05)	0.04 (0.08)	–	0.40 (3.60)	0.48 (1.20)	-0.38 (2.08)	0.01 (0.07)
	0.16 (0.08)*	0.02 (0.05)	–	0.98 (0.82)**	0.56 (0.57)	0.80 (1.08)	0.14 (0.08)*
rp	0.08 (0.08)	-0.01 (0.06)	-0.03 (0.08)	–	-0.01 (0.43)	0.53 (1.52)	0.10 (0.07)
	0.14 (0.06)*	-0.0004 (0.04)	0.13 (0.06)*	–	0.30 (0.22)	0.92 (0.13)**	0.18 (0.06)**
e12	0.24 (0.06)***	0.37 (0.09)***	0.03 (0.06)	0.004 (0.04)	–	0.89 (0.40)***	0.56 (0.06)***
	0.18 (0.06)**	0.23 (0.08)***	0.11 (0.07)	0.06 (0.04)	–	0.48 (0.26)*	0.30 (0.08)***
e12a	0.22 (0.12)**	0.20 (0.07)***	-0.04 (0.07)	0.09 (0.09)	0.20 (0.06)***	–	0.15 (0.08)*
	0.22 (0.10)*	0.04 (0.05)	0.16 (0.10)	0.17 (0.08)*	0.12 (0.06)*	–	0.16 (0.08)*

Note: The abbreviations used for the life-history traits are described in the text of Materials and Methods section. Genetic covariances are shown below diagonal and genetic correlations above diagonal. In the top line (of each trait combination) are values for populations of A. nanus originating from the habitat C, whereas the values for the populations from habitat S are in the bottom line. SE are given in parentheses. ***P < 0.001, **P < 0.01, *P < 0.05 indicate significance level when different from 0.

G matrices

Analysis of effective dimensionality of **G** matrices performed with the use of factor-analytic approach separately for each habitat, indicated the best fit of the two-dimensional model in both instances. Likelihood ratio tests of the nested hypotheses showed for both habitats the first significant increase in the log-likelihood, when changing the two-dimensional covariance structure to one-dimensional structure ($\chi^2 = 17.7$, df = 5, $P < 0.0033$ and $\chi^2 = 13.3$, df = 5, $P < 0.021$; for habitat C and habitat S, respectively). The best fit of two-dimensional genetic covariance structure for the populations from habitat S was supported by the lowest Akaike information criteria (AIC) value (Table 2). For the populations from habitat C, the model implementing three-dimensional structure returned the lowest AIC value. However, this value did not differ significantly from the AIC of the two-dimensional structure.
The subsequent analysis used two-dimensional covariance structure, FA0(2), to test for the differences between the reduced-rank matrices of the populations from different habitats. The likelihood ratio test indicated a significant difference between these matrices ($\chi^2 = 34.6$, df = 11, $P = 0.0003$). The same conclusion could be derived from the inspection of AIC: the reduced model resulted in AIC = 3993.6 while the full model in AIC = 4006.2.

*Table 2: Fit statistics for the nested series of factor-analytic models testing the dimensionality of **G** matrices in both habitats.*

	Habitat C		Habitat S		No. of covariance parameters
	-2LL	AIC	-2LL	AIC	
FA0(6)	1525.6	1607.6	2204.8	2286.8	21
FA0(5)	1525.6	1605.6	2204.6	2286.6	20
FA0(4)	1525.6	1603.6	2205.1	2283.1	18
FA0(3)	1529.8	1601.8	2206.7	2278.7	15
FA0(2)	1538.0	1602.0	2211.0	2275.0	11
FA0(1)	1555.7	1609.7	2224.3	2278.3	6

Note: Tests for habitat S and habitat C performed separately; LL stands for log likelihood and AIC for Akaike information criterion.

Comparison of **G** matrices between the habitats (pooled across replicate populations) using Flury hierarchy indicated a low level of shared structure. The hypothesis of a single common principal component was not ruled out (Table 3) while the hypothesis of two shared principal components was rejected ($P = 0.013$). These substantial changes in **G** matrices (Fig. 2) can most likely be attributed to the applied

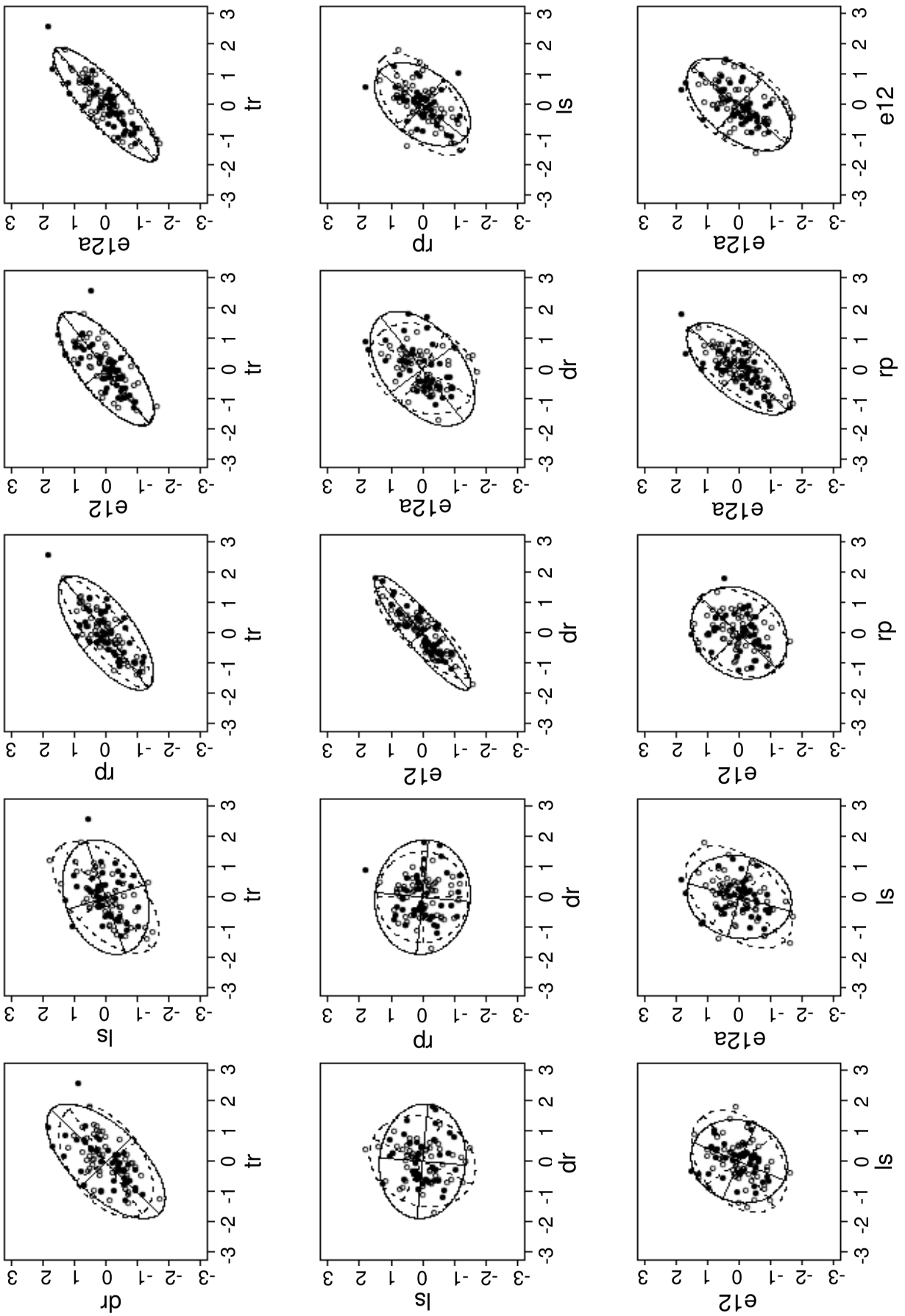


Figure legend on the next page

divergent selective pressure. When four replicate populations within habitat S were compared, the verdict of all common principal components was reached (i.e. the hypothesis of shared all principal components 'CPC' was not rejected (Table 3), while the hypothesis of proportionality was declined $P = 0.011$). This indicates relatively less divergence among the replicate plots than between the habitats. Corresponding comparison of six-by-six genetic matrices for habitat C could not be carried out because the initial **G** matrix had too many negative eigenvalues. Since the traits with little genetic variance can be responsible for such situation, two traits which showed non-significant heritability values (i.e. life-span and reproductive period) were excluded from the analysis. The resulting four-trait matrices showed unrelated structure across the replicate populations within habitat C (Table 3), indicating high variation in genetic structure.

Environmental and phenotypic (co)variance matrices

Pooled environmental variance-covariance matrices (**E**) showed unrelated structure between habitats (Table 3). The hypothesis of a single shared principal component was rejected ($P = 0.006$). In contrast, **E** matrices of the replicate populations within both habitats showed a considerable level of similarity. Namely, Flury hierarchy indicated that equality hypothesis could not be rejected for **E** matrices of replicate populations

*Table 3: Comparisons of **G**, **E** and **P** matrices with Flury hierarchy.*

	G similarity (*P*)	**E** similarity (*P*)	**P** similarity (*P*)
Between habitats	CPC1 (0.14)	Unrelated	Unrelated
Among populations within			
Habitat C	Unrelated	Equal (0.13)	CPC (0.19)
Habitat S	CPC (0.39)	CPC3 (0.59)	Equal (0.41)

Note: Results are based on 10000 randomizations over clones (*CPCrand* software). The *P*-values indicate that the similarity hypothesis could not be rejected.

Figure 2: Genetic covariances of life-history traits for the populations of A. nanus from habitat C (solid lines) and from habitat S (dashed lines). Each graph represents different trait combination. Ellipses of 95% confidence interval were calculated based on the standardized means (overall mean=0; SD=1) for clones pooled within habitat type. Principal components of the matrices were used to orientate the ellipses in the plane. The ellipses were centered through the origin of principal axes. The clones' means are represented by solid circles for habitat C and open circles for habitat S. Note that each graph represents a bivariate plane which is a part of six-dimensional data set.

within habitats C ($P = 0.13$). For habitat S the hypothesis of three CPC could not be ruled out ($P = 0.59$). Pooled phenotypic matrices showed less similarity across the habitats than the corresponding **G** matrices. In fact, the hypothesis of a single shared principal component was rejected ($P = 0.032$) indicating the unrelated structure of the matrices. The variation among **P** matrices within habitat S was lower than within habitat C; the analysis indicated all common principal components in the matrices in habitat C ('CPC'; $P = 0.19$), while in habitat S the hypothesis of equality could not be rejected ($P = 0.41$). In general, **P** and **E** matrices showed less divergent structure within habitat than between habitats.

Discussion

We demonstrated a rapid divergence (20 years) of **G** structure within a natural population of the parthenogenetic nematode, *A. nanus*, in response to the imposed divergent selection and drift. To our knowledge, this study is the first attempt to combine the realism of field situation with the control of an evolution experiment, where the selective agent and evolutionary time-frame is defined by experimental manipulation. The documented examples of **G** matrix evolution in natural populations consider commonly the time-spans of thousands or millions of years (Arnold & Phillips 1999; Cano *et al.* 2004). The time of divergence of genetic architecture reported in this study is more comparable with the ones observed in laboratory studies (Bryant & Meffert 1988; Wilkinson *et al.* 1990; Shaw *et al.* 1995; Phillips *et al.* 2001). We are not aware of any other reports of such high dynamics of **G** structure (involving non-transient changes) in natural populations. Therefore, we have a reason to believe that there might be a major difference between our and the other analysed natural systems. The obvious characteristics distinguishing *A. nanus* from the other analysed species is obligate parthenogenetic reproduction. It has been acknowledged that the mode of reproduction (recombination frequency) influences the behaviour of **G** matrices (Kelly 1999; Phillips & McGuigan 2006). The general expectation is that recombination will contribute to the stability of **G** mainly through breaking of genetic disequilibria, which otherwise would accumulate during asexual reproduction (Deng & Lynch 1996; Pfrender & Lynch 2000). It needs to be noted that genetic covariances are determined mainly by pleiotropy and linkage disequilibria, which can have different implications for evolution of genetic architecture and phenotypic characters. Namely, genetic covariances determined mainly by disequilibria, are expected to evolve more rapidly with selection than genetic covariances depending on pleiotropy (Deng & Lynch 1996; Kelly 1999). For example, in *Daphnia pulex* **G** matrices changed rapidly during prolonged clonal selection and this change could be attributed to the build-up of genetic disequilibria (Pfrender & Lynch 2000). Although the changes in **G** due to

genetic disequilibria and non-additive effects are not permanent for populations that undergo cyclically sexual reproduction, in asexuals the changes persist due to lack of recombination. Because *A. nanus* reproduces by obligate parthenogenesis the assessment of the relative contribution of both quantities to genetic correlations is difficult. Nevertheless, it can be safely assumed that the influence of genetic disequilibria on genetic covariances in *A. nanus* was high. The results of our study follow, therefore, theoretical predictions and support the general view of destabilizing effects of genetic disequilibria on **G** matrix. The results imply also that the predictive value of **G** for analysing phenotypic evolution in asexual species or species with rare recombination events might be limited.

The studies implementing analytical approach (e.g. Turelli 1988) and stochastic computer modelling (e.g. Jones *et al.* 2003) resulted in formulating a number of conditions promoting stability of **G** structure. **G** stability is expected to be enhanced by, among others, large population sizes or presence of strong correlations of mutational effects. It is also predicted that fitness components might have less stable **G** matrices. In relation to populations size, we found that the populations inhabiting a single plot (6 × 11 m), regardless of habitat type, consisted of approximately 2×10^5 – 5×10^5 individuals (unpublished data). These numbers, which are likely to be representative for effective population size (Balloux *et al.* 2003), are relatively high. Consequently, the differences in **G** among populations were most probably not enhanced by the population sizes of *A. nanus*. Our findings are also in concordance with the prediction of the reduced stability of **G** matrices of life-history traits. The influence of correlational selection and mutation effects remains unclear, since these aspects were not analysed in the used system. In general, although stability of **G** matrices in *A. nanus* could have been influenced by the mentioned factors, their role was possibly minor in comparison to the influence of the reproductive mode. It is also not entirely clear whether the predictions derived under the assumption of sexual reproduction and genetic equilibrium hold also for asexual species.

Using Flury hierarchy and factor analytic approach we showed divergence of **G** structure in *A. nanus* due to the applied divergent selection. The detected differences concern both the magnitude of matrix elements and orientation of genetic covariances and therefore are in concordance with theoretical predictions of non-proportional changes in **G** in response to strong selection (Roff 2000). The level of divergence of **G** among replicate populations was generally high and depended on the habitat type. Populations of habitat C showed higher level of divergence (unrelated **G** structure) than the populations of habitat S (verdict of common principal components). According to theoretical predictions, the lack of major differences in selective pressures across populations within the same habitat should conserve the structure of **G** and effect of drift is expected to be restricted to its proportional changes (Roff 2000). Therefore, the level of shared structure of **G** matrices of populations inhabiting

the same habitat detected in our study is lower than expected. It has been indicated, however, that proportional change in response to drift is rather an average effect and individual populations are likely to show a whole range of structural changes of **G** (Phillips *et al.* 2001; Widen *et al.* 2002). The difference in the level of divergence of replicate populations between the habitats might be related to the difference in generation time of the nematodes. In our previous study we estimated the number of generations per year as 6.6 for habitat C and 0.7 in habitat S (chapters 2 and 3, this thesis), which indicates that populations of habitat C were subjected to drift for longer 'evolutionary time'. Alternatively, the divergence within habitats might reflect not only the effect of drift but also possible effects of local selective pressures, which could have more pronounced effect on the populations of habitat C.

Factor-analytic approach showed that six analysed life-history traits in populations from both habitats could be adequately represented by two underlying genetic dimensions (two-dimensional **G** matrices) indicating a relatively high level of absolute constraints. These findings support the idea that the absolute constraints could be a common feature of traits' genetic architecture (e.g. Kirkpatrick & Lofsvold 1992; but see Mezey & Houle 2005). Our low estimate of dimensionality can be probably partly explained by the choice of the phenotypic traits. All but one of the analysed traits were related to reproduction and genetic correlations between these traits were generally high (some did not differ significantly from 1; Table 1). With multiple traits highly correlated with each other it is more likely to observe absolute constraints (Kirkpatrick & Lofsvold 1992). We chose factor-analytic approach to analyse dimensionality because of its flexibility in implementing various experimental designs and high reliability of the obtained results (Hine & Blows 2006).

The reliability of laboratory estimates of genetic parameters has been questioned in several studies (Charmantier & Garant 2005; Pigliucci 2006). Environmental influence on genetic covariance structure has been demonstrated on multiple occasions (Guntrip *et al.* 1997; Cano *et al.* 2004) and led to the general conclusions that genetic architecture is likely to breakdown in novel environments, thus also in the laboratory (Charmantier & Garant 2005). Under novel but less variable laboratory conditions, environmental variance is likely to be reduced and the expression of genetic variance might increase (Sgro & Hoffmann 1998) leading to an overestimation of heritability and the elements of **G**. Although our results might suffer from similar bias, the observed differences between the habitats are likely to remain valid. In fact, if the genetic parameters were estimated under field conditions, the detected difference could be even larger than the one based on the laboratory estimates. Stress environments are usually more variable than favourable ones (Charmantier & Garant 2005). Therefore, laboratory estimates of heritability and elements of **G** are expected to be more inflated for the populations from habitat S, what would imply that the real differences between habitats are larger than observed. The analysis with Flury

hierarchy might have also introduced some bias to the results through implementation of 'bending' procedure which is known to change the error structure of the comparison matrix. It is, however, unsure what kind of bias might be expected when this procedure is applied.

It has often been suggested that phenotypic matrices are reliable estimation of **G** (Steppan *et al.* 2002). Although we did not compare **P** with **G** directly, our results show that the response of phenotypic matrices to the imposed selection and drift differed from the one found for **G** matrices. Consequently, we are of the opinion that the interpretation of the patterns of **P** as representative for genetic matrices should be treated with caution.

Understanding patterns of phenotypic variation produced by selection and ability to make predictions of future phenotypic responses depends on our knowledge of the stability of genetic variance-covariance matrices. The information on how the structure of **G** evolves and what conditions promote these changes would allow to decide whether an evolution analysis with the breeder's equation could be performed (Phillips & McGuigan 2006). The high dynamics of the changes in genetic architecture reported in this study is in evident contrast with most of the existing studies on natural populations. In our opinion, this difference is mainly due to asexual reproduction of *A. nanus* and resulting accumulation of genetic disequilibria. Structure of genetic variance-covariance in asexual species or the species with rare events of sexual reproduction is likely to respond rapidly to selection and drift and therefore the predictive power of **G** for their phenotypic evolution might be limited.

Acknowledgements

We thank A.W.G. van der Wurff and B. Sheldon for their valuable comments on the previous version of the manuscript and M. Blows for the advice on the factor-analytic method. The work was supported by the TRIAS Programme (TRIpartite Approach to Soil system processes) of Delft Cluster, SKB (Foundation for development and transmission of soil knowledge) and NWO (the Netherlands Organisation for Scientific Research), as project 835.80.008.

References

Arnold, S.J. & Phillips, P.C. (1999) Hierarchical comparison of genetic variance-covariance matrices. II. Coastal-inland divergence in the garter snake, *Thamnophis elegans*. *Evolution*, **53**, 1516-1527.

Balloux, F., Lehmann, L. & de Meeus, T. (2003) The population genetics of clonal and partially clonal diploids. *Genetics*, **164**, 1635-1644.

Bird, A.F., De Ley, P. & Bird, J. (1993) Morphology, oviposition and embryogenesis in an Australian population of *Acrobeloides nanus*. *Journal of Nematology*, **25**, 607-615.

Bjorklund, M. (1996) The importance of evolutionary constraints in ecological time scales. *Evolutionary Ecology*, **10**, 423-431.

Bonting, C.F.C., Willemsen, B.M.F., Akkermansvanvliet, W., Bouvet, P.J.M., Kortstee, G.J.J. & Zehnder, A.J.B. (1992) Additional characteristics of the polyphosphate-accumulating *Acinetobacter* strain 210a and its identification as *Acinetobacter johnsonii*. *FEMS Microbiology Ecology*, **102**, 57-64.

Bryant, E.H. & Meffert, L.M. (1988) Effect of an experimental bottleneck on morphological integration in the housefly. *Evolution*, **42**, 698-707.

Cano, J.M., Laurila, A., Palo, J. & Merila, J. (2004) Population differentiation in G matrix structure due to natural selection in *Rana temporaria*. *Evolution*, **58**, 2013-2020.

Charmantier, A. & Garant, D. (2005) Environmental quality and evolutionary potential: lessons from wild populations. *Proceedings of the Royal Society B: Biological Sciences*, **272**, 1415-1425.

Cheetham, A.H., Jackson, J.B.C. & Hayek, L.A.C. (1994) Quantitative genetics of Bryozoan phenotypic evolution .2. Analysis of selection and random change in fossil species using reconstructed genetic parameters. *Evolution*, **48**, 360-375.

De Goede, R.G.M. & Bongers, T. (1998) *Nematode communities of northern temperate grassland ecosystems* Focus, Giessen.

Deng, H.W. & Lynch, M. (1996) Change of genetic architecture in response to sex. *Genetics*, **143**, 203-212.

Guntrip, J., Sibly, R.M. & Holloway, G.J. (1997) The effect of novel environment and sex on the additive genetic variation and covariation in and between emergence body weight and development period in the cowpea weevil, *Callosobruchus maculatus* (Coleoptera, Bruchidae). *Heredity*, **78**, 158-165.

Hine, E. & Blows, M.W. (2006) Determining the effective dimensionality of the genetic variance-covariance matrix. *Genetics*, **173**, 1135-1144.

Jones, A.G., Arnold, S.J. & Borger, R. (2003) Stability of the G-matrix in a population experiencing pleiotropic mutation, stabilizing selection, and genetic drift. *Evolution*, **57**, 1747-1760.

Jones, A.G., Arnold, S.J. & Burger, R. (2004) Evolution and stability of the G-matrix on a landscape with a moving optimum. *Evolution*, **58**, 1639-1654.

Kelly, J.K. (1999) Response to selection in partially self-fertilizing populations. II. Selection on multiple traits. *Evolution*, **53**, 350-357.

Kirkpatrick, M. & Lofsvold, D. (1992) Measuring selection and constraint in the evolution of growth. *Evolution*, **46**, 954-971.

Kirkpatrick, M. & Meyer, K. (2004) Direct estimation of genetic principal components: Simplified analysis of complex phenotypes. *Genetics*, **168**, 2295-2306.

Korthals, G.W., Alexiev, A.D., Lexmond, T.M., Kammenga, J.E. & Bongers, A.M.T. (1996) Long-term effects of copper and pH on the nematode community in an agroecosystem. *Environmental Toxicology and Chemistry*, **15**, 979-985.

Lande, R. (1979) Quantitative genetic analysis of multivariate evolution, applied to brain - body size allometry. *Evolution*, **33**, 402-416.

Lande, R. (1980) The genetic covariance between characters maintained by pleiotropic mutations. *Genetics*, **94**, 203-215.

Lande, R. & Arnold, S.J. (1983) The measurement of selection on correlated characters. *Evolution*, **37**, 1210-1226.

Laugsch, M. & Schierenberg, E. (2004) Differences in maternal supply and early development of closely related nematode species. *International Journal of Developmental Biology*, **48**, 655-662.

Lynch, M. & Walsh, J.B. (1998) *Genetics and Analysis of Quantitative Traits* Sinauer Associates, Sunderland, MA.

Marroig, G. & Cheverud, J.M. (2004) Did natural selection or genetic drift produce the cranial diversification of neotropical monkeys? *American Naturalist*, **163**, 417-428.

McGuigan, K. (2006) Studying phenotypic evolution using multivariate quantitative genetics. *Molecular Ecology*, **15**, 883-896.

Mezey, J.G. & Houle, D. (2005) The dimensionality of genetic variation for wing shape in Drosophila melanogaster. *Evolution*, **59**, 1027-1038.

Oostenbrink, M. (1960) Estimating nematode populations by some selected methods. *Nematology* (eds J.N. Sasser & W.R. Jenkins), pp. 85-102. The University of North Carolina Press, Chapel Hill, NC.

Paulsen, S.M. (1996) Quantitative genetics of the wing color pattern in the buckeye butterfly (*Precis coenia* and *Precis evarete*): Evidence against the constancy of G. *Evolution*, **50**, 1585-1597.

Pfrender, M.E. & Lynch, M. (2000) Quantitative genetic variation in *Daphnia*: Temporal changes in genetic architecture. *Evolution*, **54**, 1502-1509.

Phillips, P.C. & Arnold, S.J. (1999) Hierarchical comparison of genetic variance-covariance matrices. I. Using the Flury hierarchy. *Evolution*, **53**, 1506-1515.

Phillips, P.C., Whitlock, M.C. & Fowler, K. (2001) Inbreeding changes the shape of the genetic covariance matrix in *Drosophila melanogaster*. *Genetics*, **158**, 1137-1145.

Phillips, P.C. & McGuigan, K.L. (2006) Evolution of genetic variance-covariance structure. *Evolutionary genetics, concepts and case studies* (eds C.W. Fox & J.B. Wolf). Oxford University Press, Oxford.

Pigliucci, M. (2006) Genetic variance-covariance matrices: A critique of the evolutionary quantitative genetics research program. *Biology and Philosophy*, **21**, 1-23.

Roff, D. (2000) The evolution of the G matrix: selection or drift? *Heredity*, **84**, 135-142.

Roff, D.A. & Mousseau, T.A. (1999) Does natural selection alter genetic architecture? An evaluation of quantitative genetic variation among populations of *Allonemobius socius* and *A. fasciatus*. *Journal of Evolutionary Biology*, **12**, 361-369.

Roff, D.A., Mousseau, T.A. & Howard, D.J. (1999) Variation in genetic architecture of calling song among populations of *Allonemobius socius, A. fasciatus*, and a hybrid population: Drift or selection? *Evolution*, **53**, 216-224.

Sgro, C.M. & Hoffmann, A.A. (1998) Effects of stress combinations on the expression of additive genetic variation for fecundity in *Drosophila melanogaster*. *Genetical Research*, **72**, 13-18.

Shaw, F.H., Shaw, R.G., Wilkinson, G.S. & Turelli, M. (1995) Changes in genetic variances and covariances: G whiz! *Evolution*, **49**, 1260-1267.

Sohlenius, B. & Boström, S. (1999) Effects of global warming on nematode diversity in a Swedish tundra soil - a soil transplantation experiment. *Nematology*, 695-709.

Steppan, S.J., Phillips, P.C. & Houle, D. (2002) Comparative quantitative genetics: evolution of the G matrix. *Trends in Ecology and Evolution*, **17**, 320-327.

Turelli, M. (1988) Phenotypic evolution, constant covariances, and the maintenance of additive variance. *Evolution*, **42**, 1342-1347.

Widen, B., Andersson, S., Rao, G.Y. & Widen, M. (2002) Population divergence of genetic (co)variance matrices in a subdivided plant species, *Brassica cretica*. *Journal of Evolutionary Biology*, **15**, 961-970.

Wiegner, O. & Schierenberg, E. (1998) Specification of gut cell fate differs significantly between the nematodes *Acrobeloides nanus* and *Caenorhabditis elegans*. *Developmental Biology*, **204**, 3-14.

Wilkinson, G.S., Fowler, K. & Partridge, L. (1990) Resistance of genetic correlation structure to directional selection in *Drosophila melanogaster*. *Evolution*, **44**, 1990-2003.

Chapter 5

Genomic library of CB4856 / N2 near-isogenic lines of *Caenorhabditis elegans* and mapping of aggregation behaviour

Agnieszka Doroszuk
Emilie F. Fradin
Bas van de Waterbeemd
Joost A.G. Riksen
Ana Quilez-Moraga
Jan E. Kammenga

Manuscript in preparation

Abstract

There has been a growing interest in the application of QTL methods in *Caenorhabditis elegans* research over the last years. The progress remains, however, hampered by the lack of powerful resources allowing efficient QTL identification and their precise localization. We have constructed a library of 87 near-isogenic lines (NILs), each carrying a single homozygous genome segment of the Hawaiian strain CB4856 introgressed into the genetic background of the Bristol strain N2, as determined by 121 single nucleotide polymorphism (SNP) markers. The proportion of the CB4856 segments in most lines does not exceed 3% and together the introgressions provide a coverage of approximately 94% of the total genome. The utility of the created NIL library was demonstrated by identifying novel loci underlying natural variation in aggregation behaviour. The initial genome-wide scan using recombinant inbred lines (RILs) identified 3 candidate QTLs, on chromosome III (agr3), IV (agr4) and X (agrX). The NIL analysis localized agr3, a novel locus for this phenotype, to a 8.7 cM segment. The position and effect of agrX determined with NILs corresponded with the position of *npr*-1, the only gene known to influence natural variation in *C. elegans* aggregation. The NIL approach did not confirm the presence of agr4. Overall, the presented NIL library provides the first step towards easier and efficient fine-mapping and functional analyses of loci underlying complex traits in *C. elegans*.

Introduction

Over the last decades, *Caenorhabditis elegans* has become a successful model organism for unravelling genetic mechanisms underlying many traits such as responses to stress, ageing, host-pathogen interaction, behaviour, learning and memory (Kurz & Ewbank 2003; Gotz *et al.* 2004; de Bono & Maricq 2005; Gami & Wolkow 2006; Giles *et al.* 2006). Gene discovery in *C. elegans* is predominantly based on the forward genetic methods facilitated by the ease of mutagenesis and mutant analysis. Recently, more attention has been directed towards an alternative approach: quantitative trait locus (QTL) analysis, a strategy, which focuses on identification of natural variation of complex traits. QTL analysis has been already applied in *C. elegans* to study life-history traits (Shook & Johnson 1999b; Ayyadevara *et al.* 2001; Gutteling *et al.* 2006) and sensitivity to volatile anaesthetics (Van Swinderen et al. 1997). Using this approach, it was also possible to investigate phenotypic plasticity, pleiotropy, genotype × environment interactions and epistasis in *C. elegans* life-histories (Shook *et al.* 1996; Shook & Johnson 1999a; Ayyadevara *et al.* 2001; Ayyadevara *et al.* 2003; Gutteling *et al.* 2006).

Both approaches, mutagenesis and QTL mapping, have their advantages as well as shortcomings and they bring unique insights into complex phenotypes (Flint & Mott 2001; Korstanje & Paigen 2002). Mutant screens that rely mostly on large genetic effects, provide important information for pathway analysis, but are of limited value in determining loci, which underlie the variety of phenotypes in natural populations (Mackay 2001; Ayyadevara *et al.* 2003). There is also an important consequence of using either of the methods for studying the genetics of diseases: while mutagenesis results predominantly in detection of genes in a pathway resembling rare mutations causing a disease, QTL mapping is expected to identify genes encoding rate-limiting enzymes or regulatory proteins which might serve as future therapeutic targets (Korstanje & Paigen 2002).

QTL detection depends on many factors such as density of marker loci, heritability of the examined trait and the type and size of mapping population. Recently, considerable advancements have been made in the development of QTL mapping resources such as recombinant inbred lines (RILs), advanced intercross lines (AILs), heterogeneous stock (HS) or near-isogenic lines (NILs) for many model and economically important species (Darvasi & Soller 1995; Eshed & Zamir 1995; Iakoubova *et al.* 2001; Davis *et al.* 2005; Eduardo *et al.* 2005; Li *et al.* 2005; Pestsova *et al.* 2006; Valdar *et al.* 2006). Surprisingly, there has been no similar achievements in the construction of *C. elegans* mapping populations. Mapping studies involving *C. elegans* use mainly RILs derived from various genetically distinguishable parental strains. Their genetic constitution was, in most cases, determined with transposon elements Tc1, the markers of rather small density and with limited genome coverage (but see Gutteling *et al.* 2006). In some cases, small sets of near-isogenic lines (NILs)

were constructed to fine-map QTLs previously detected with RIL populations (Knight *et al.* 2001; Ayyadevara *et al.* 2003). Overall, locating QTLs with the accuracy, which would allow identification of a reasonable number of candidate genes in *C. elegans* remains difficult with current resources.

A strategy that proved to be successful in efficient and precise determination of QTL positions is the use of NIL libraries (Paran & Zamir 2003; Flint *et al.* 2005). In principle, the genome of a near-isogenic line (which is also referred to as a congenic strain or an introgression line) is composed of the recipient (or background) genome contributed by one of the parental strains and a short, homozygous segment of the donor genome contributed by another, genetically distinct, parental strain. In this way, the difference in phenotype of the recipient (background) strain and the NIL can be precisely attributed to the introgressed segment. Without the confounding effects of other segregating QTLs, the effect of the locus of interest can be determined with high accuracy. The additional value of NIL libraries is that the selected NILs can be further backcrossed to the recipient parent and the resulting set of lines with shorter introgressions (sub-NILs) can be used to provide high-resolution information on QTL architecture. In rice and tomato, NILs were successfully applied to dissect QTLs of large effects into multiple, tightly linked loci (Fridman *et al.* 2002; Thomson *et al.* 2006). High-resolution mapping using NIL proved also effective in gene identification and facilitated subsequent positional cloning (Clark *et al.* 2006; Clee *et al.* 2006) providing clues for the molecular basis of gene functions.

We report the development of a library of near-isogenic lines of *C. elegans* from the parental strains Bristol N2 and Hawaiian CB4856. The canonical N2 strain was chosen as the recipient (background) genome and CB4856 as the donor genome. Many phenotypic and genetic differences between these strains, including single nucleotide polymorphisms (SNP) used in this study as genetic markers, have been described (Koch *et al.* 2000; Swan *et al.* 2002) and both strains are broadly used in *C. elegans* genetic research.

To demonstrate the usefulness of NILs in QTL mapping, we aimed to identify novel loci associated with aggregation behaviour. Aggregation behaviour (which is also referred to as social feeding or clumping behaviour) is differentially displayed by the parental strains (CB4856 aggregates while N2 is solitary) and it represents a form of stress reaction (de Bono *et al.* 2002; Chang *et al.* 2006). The only gene known to contribute to natural variation in *C. elegans* aggregation is *npr-1*, which was found to show different isoforms in solitary and aggregating wild isolates (de Bono & Bargmann 1998). In this study, we apply the approach of an initial genome-wide scan with an established RIL population (Li *et al.* 2006), which is followed by the examination of the regions comprising putative QTLs with the use of a subset of the created NIL library.

Results

Construction and characterization of NIL population

We chose a set of eight recombinant inbred lines (RILs): WN17, WN19, WN28, WN29, WN57, WN64, WN77, WN80 (Li *et al.* 2006) as the basis for the construction of the near-isogenic lines (NIL). The selected RILs were part of a RIL library consisting of 80 fully genotyped lines derived from the cross between the strains CB4856 and N2. We selected RILs on the basis of the percentage and location of the donor genome (CB4856), as determined by 121 single nucleotide polymorphisms (SNP) markers (Li *et al.* 2006). Each chosen RIL contained a low proportion (~30%) of donor segments, while together the CB4856 segments covered the whole genome length. Depending on the proportion of the donor genome, RILs underwent 3-7 generations of backcrossing to the recurrent parent, N2, followed by 10 generations of selfing and subsequent determination of 121 SNP markers (Li *et al.* 2006). The detailed description of the breeding scheme is presented in the Methods section and is illustrated in Figure 1.

We constructed 87 NILs, each containing a single, homozygous genomic segment of CB4856 introgressed into the N2 genomic background (assuming no double crossing-overs between adjacent markers). Each chromosome was represented by, on average, 14.5 NILs and the length of donor segments ranged from 0.7 to 25.8 cM, which is the equivalent of 0.25 to 9.4% of the total genome length. The schematic representation of the genotypes is depicted in Figure 2. The distribution of the introgression length was right skewed, with approximately 70% of introgression shorter than 10 cM and with the median of approximately 7 cM. The donor segments in different NILs predominantly overlapped. On five locations (on chromosomes *I, II III* and *X*), however, the adjacent introgressions in two NILs were not overlapping (Fig. 2). Consequently, some part of these intervals might not be covered by introgressions. We estimated the maximum total length of the genomic segments non-covered by the introgressions as 6.9% of the total genome length. Unique overlapping regions divide the genome into bins. In the presented library, the CB4856 introgressions defined, in total, 80 bins of the average size of 3.6 cM. The average bin size for a chromosome depended on the total number of NILs constructed for it and ranged from 2.9 cM for chromosome *IV* to 5.8 for chromosome *II*. The bins in the middle parts of chromosomes (except chromosome *X*) were usually larger than in the chromosome arms, which is the consequence of lower recombination rate in the central cluster (Consortium 1998). In chromosome *X*, the bin sizes were more uniform across all the chromosome length, which is consistent with the observations of lower variation in recombination frequency across its length (Consortium 1998). Overall, the overlaps among NILs have potential to increase the mapping resolution to 3-4 cM.

Aggregation in RILs

Initially, using a set of 70 RILs (Li *et al.* 2006) and the parental strains, we performed a genome-wide scan of this phenotype. CB4856 showed more pronounced aggregation behaviour than N2 (t test for independent samples, t = -5.26, df = 21, $P < 0.001$), what complies with the findings of the previous studies (De Bono & Bargmann 1998). The observation of transgressive variation in both directions in the RIL population suggested the presence of alleles reducing or enhancing clumping behaviour in both parental lines. Since the distribution of the trait departed from normality (Kolmogorov-Smirnov test, $P < 0.001$), non-parametric tests were applied to verify the results obtained with parametric tests. Using this approach, we found significant differences among RILs (ANOVA; $F_{68, 71} = 7.72$, $P < 0.001$; Kruskal-Wallis test: $\chi^2 = 97.92$, df = 69, $P = 0.013$).

Table 1: Location, significance, effects and proportion of the explained genetic variance (R^2) of QTLs for aggregation behaviour obtained with composite interval mapping and Kruskal-Wallis test using RILs.

QTL	chromosome	marker	CIM (LOD)	KW	additive effect	R^2
agr3	*III*	egPC302	3.85	14.9 ***	0.11	0.16
agr4	*IV*	pkP4051	3.41	5.35 *	0.14	0.18
agrX	*X*	pkP6120	3.63	1.05 ns	-0.1	0.14

Note: R^2 values obtained with CIM. For KW test, test statistic is shown with its significance level: * indicates $P < 0.05$; ** indicates $P < 0.01$ and *** indicates $P < 0.001$.

In general, QTLs detected by composite interval mapping procedure corresponded with the QTLs identified with Kruskal-Wallis (KW) tests. In total, three QTLs, on chromosomes *III* (agr3), *IV* (agr4) and *X* (agrX), were significantly associated with clumping behaviour (Fig. 3). Table 1 presents the nearby markers detected by KW test as well as the corresponding LOD values, the QTL effects and contribution to the genetic variance obtained with CIM procedure. The markers with the strongest association with aggregation according to KW test were also indicated as the most relevant in CIM analysis. However, the putative QTL identified on chromosome *X*, which is likely to correspond to the known *npr-1* natural variation, was found insignificant by KW test (Table 1). Clumping behaviour was more pronounced with N2 alleles at agr3 and agr4 and with CB4856 alleles at agrX. The analysis of epistatic effects among the putative QTLs, which was performed by the search of two-way interactions, did not indicate any significant interactive effects for any of the tested pairs of markers (in all cases, Dunn-Šidak adjusted $P > 0.3$).

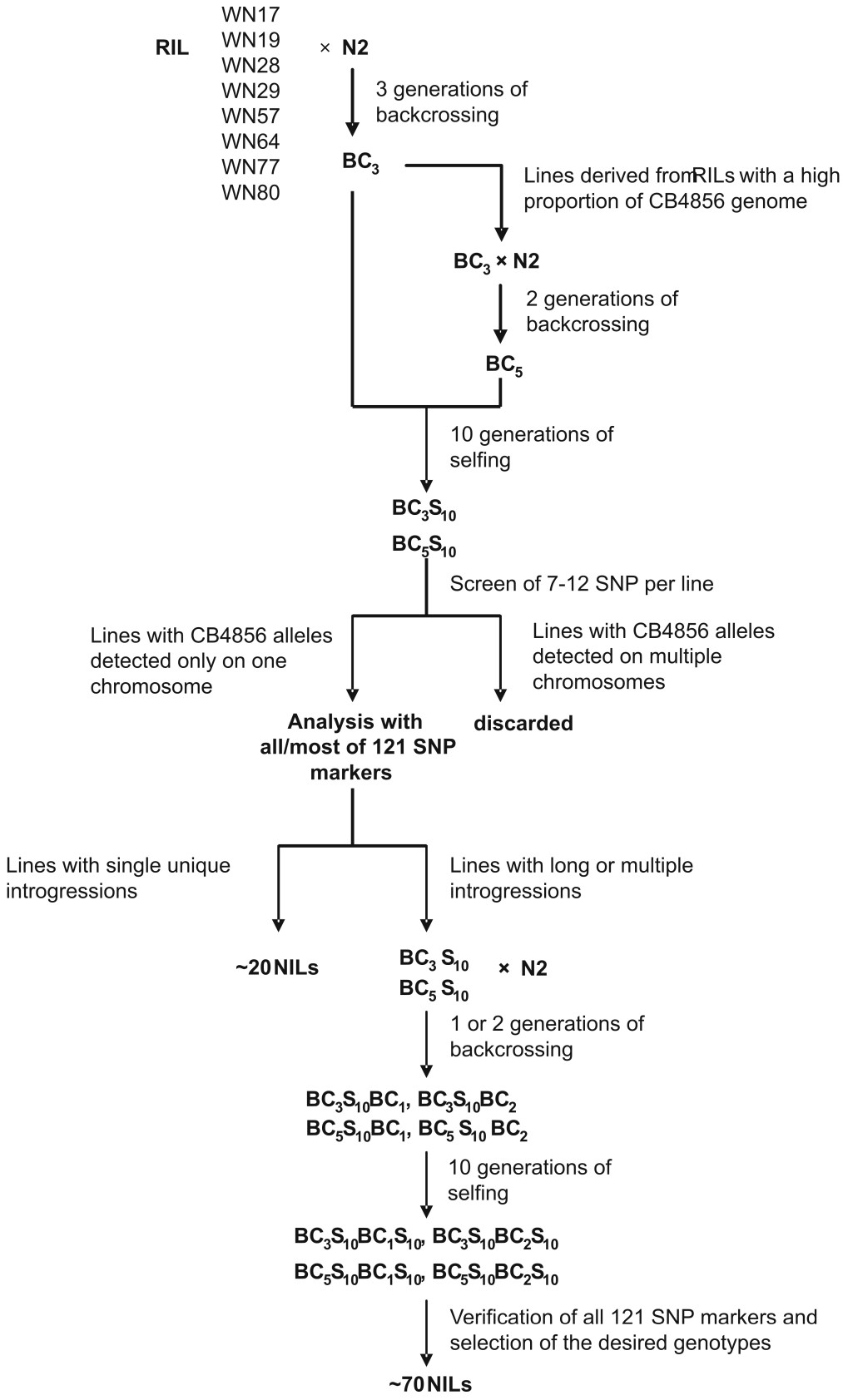


Figure 1: Breeding scheme of the C. elegans near-isogenic lines library.

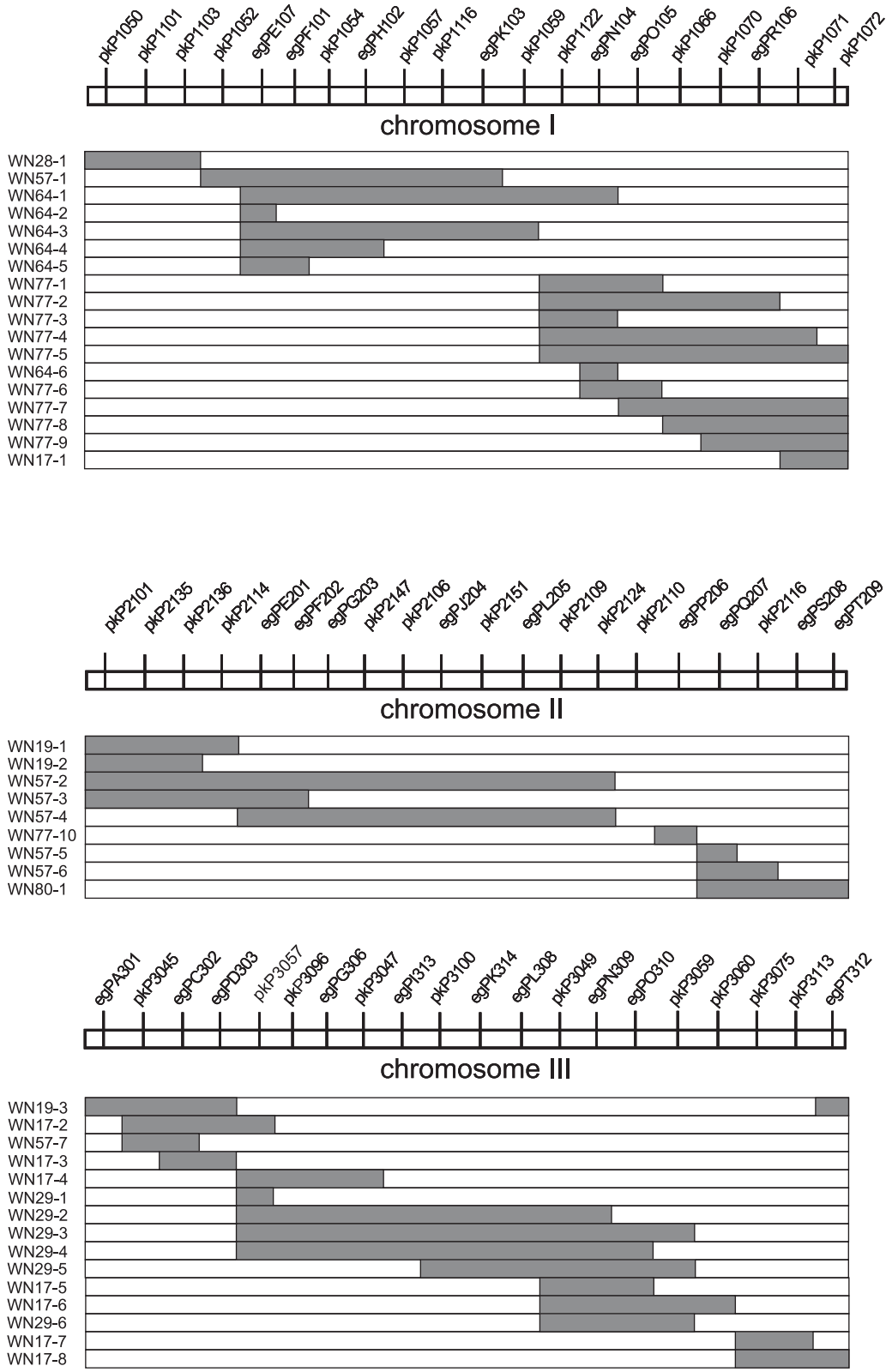


Figure 2: *Graphical representation of the NILs' genotypes, as identified with 121 SNP markers. For each line, only the chromosome carrying the introgressed CB4856 segment (grey area) is depicted. The remaining 5 chromosomes contain no donor segments. The distances between the indicated markers do not represent the real intervals (for the exact locations of the markers, see Appendix 1).*

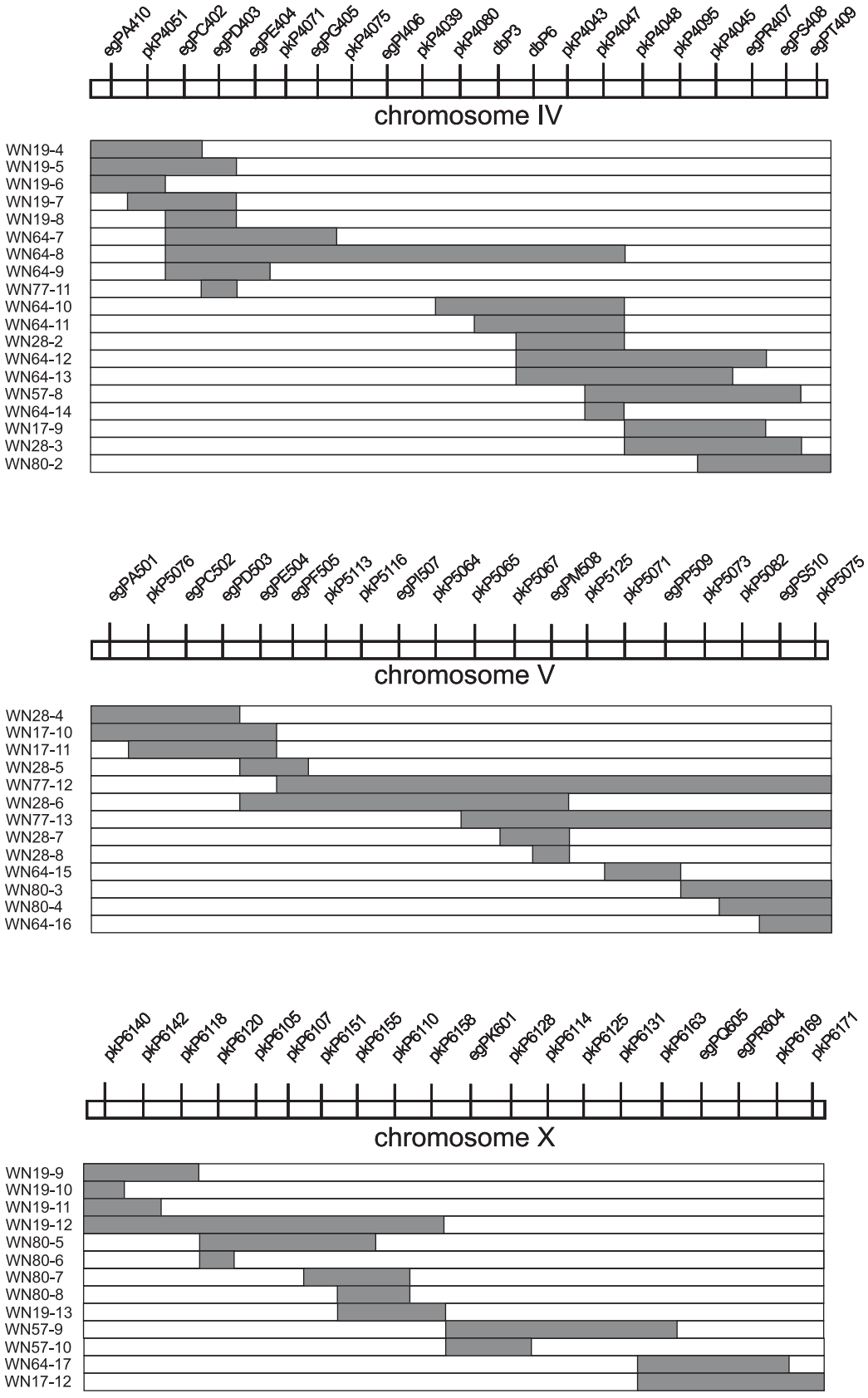


Figure legend on the previous page

Stepwise multiple regression with the peak markers of the three putative QTLs as independent variables identified agr3 as the locus with the strongest association with clumping behaviour. This locus accounted for approximately 13% of total variance while agrX was responsible for approximately 5% of total variance.

Aggregation in NILs

In order to confirm and fine-map the QTLs detected with the genome-wide scan, we conducted the analysis of 19 NILs comprising overlapping donor chromosomal segments in the detected QTL intervals. Figure 3 presents the positions of donor segments in the used NILs. ANOVA indicated significant differences among NILs ($F_{18, 151} = 15.62$, $P < 0.001$). In total, five NILs displayed stronger clumping behaviour than the N2 parental line (significant after Dunn-Šidak correction). The minimum intervals containing QTLs were determined by identifying the common region across all NILs with donor segments significantly associated with clumping. In general, the NIL analysis confirmed the approximate location of agr3 identified with CIM. Namely, three out of four near-isogenic lines with donor segments covering the region between the proximal end of chromosome *III* and the marker egPD303 aggregated more than N2 while none of the lines with introgressions spanning from the marker pkP3057 towards the middle part of the chromosome showed aggregation (Fig. 3A). The obtained results suggest, that the locus is likely to be located between the markers egPC302 and pkP3057 within the interval of 8.7 cM. Concerning chromosome IV, only one NIL (WN19-6) out of seven with donor segments covering agr4 showed significantly stronger aggregation than N2 (Fig.3B). Because in the NIL with enhanced clumping behaviour no unique donor segments were present, these results do not provide the sufficient evidence to confirm the presence of the putative agr4 in the position identified by CIM. The analysis of NILs for the proximal part of the X chromosome confirmed the presence of agrX. While all NILs with CB4856 segments introgressed between the markers pkP6140 and pkP6118 showed no significant enhancement of clumping, the line WN80-5 with the donor segment spreading between pkP6120 and pkP6110 displayed a strong clumping behaviour (Fig. 3C), indicating the location of agrX within the region flanked by these markers (9.6 cM).

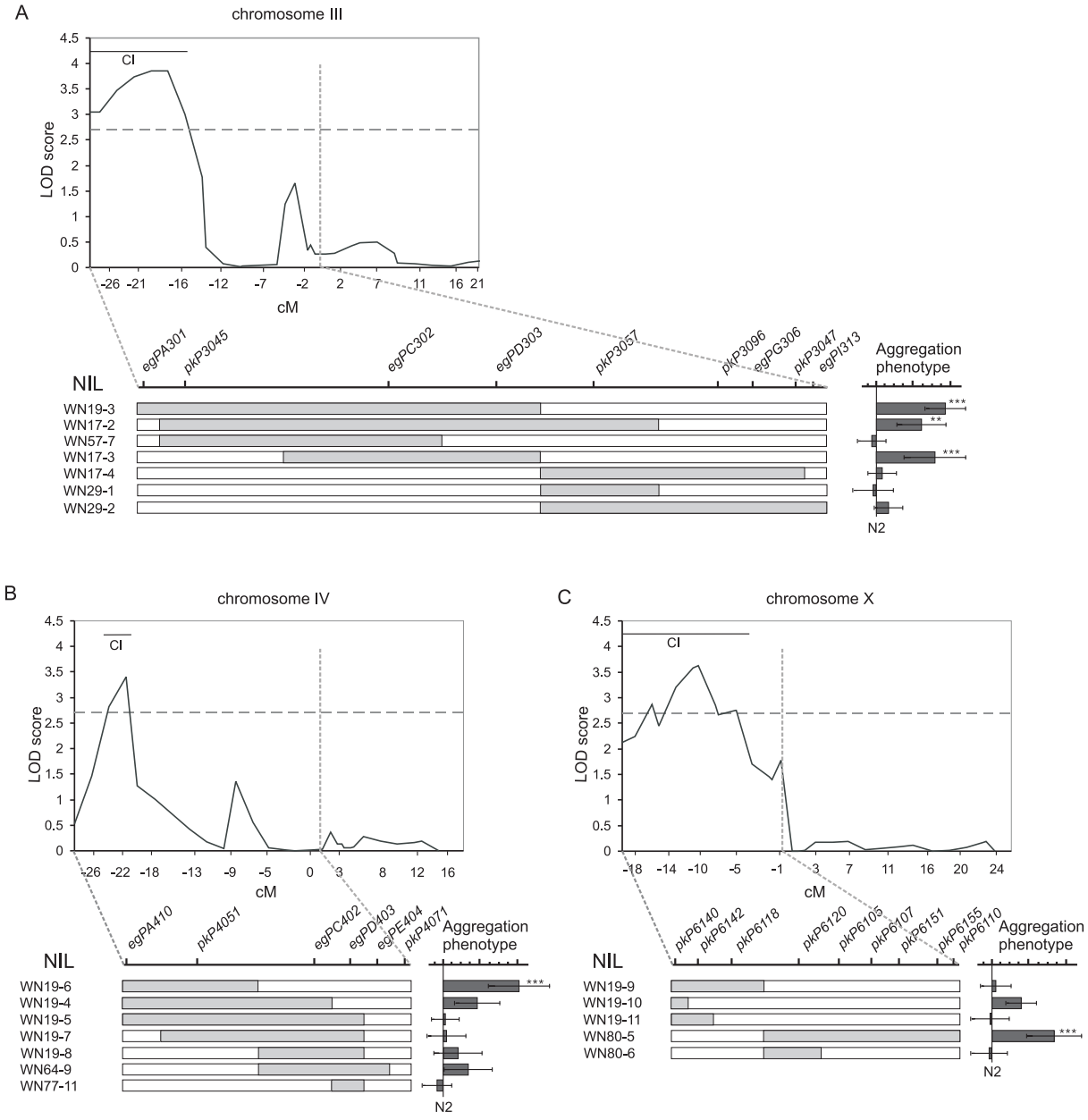


Figure 3: Mapping of aggregation behaviour using RIL mapping population and fine-mapping with NILs for chromosome III (A), chromosome IV (B) and chromosome X (C). Upper part of each panel shows QTLs with their confidence intervals (CI) found with composite interval mapping using RILs. Lower parts of the panels depict NILs used for fine-mapping (the introgressed segments are indicated by grey area) and their phenotypes relative to the phenotype displayed by the N2 parental strain (error bars indicate standard deviation).

** indicates P < 0.05; ** indicates P <0.01 and *** indicates P < 0.001 after the Dunn Šidak correction for multiple testing.*

Materials and methods

Breeding scheme

Because of the short generation-time and small body-size of the nematodes, marker assisted selection approach was difficult to perform and an alternative procedure was applied (Fig. 1). Initially, three hermaphrodites per selected RIL (WN17, WN19, WN28, WN29, WN57, WN64, WN77, WN80) were backcrossed to N2 males (six males per hermaphrodite). The BC_1 hermaphrodites from successful crosses (recognized by the male frequency in the offspring of approximately 0.5), were subsequently backcrossed to N2 males (three hermaphrodites per cross, six males per hermaphrodite). The backcrossing procedure continued with consecutive crosses of BC_2 hermaphrodites with N2 males. For the backcrosses, which were started with the RILs carrying lower proportion of donor segments, the procedure was finished with obtaining BC_3 population, while in the case of parental RILs with higher proportions of the donor genome, the BC_5 population was obtained. Subsequently, the obtained hermaphrodites were put through ten generations of selfing to create BC_3-BC_5S_{10} homozygous lines (on average 84 lines per RIL). In order to reduce the amount of genotyping work, all developed homozygous lines were initially subjected to a rough genotype screening, where only 7-12 single nucleotide polymorphism (SNP) markers distributed evenly over the donor segments (previously identified in their parental RIL), were analyzed. All lines, for which CB4856 marker alleles were identified for more than one chromosome, were discarded. The remaining lines (approximately 15 lines per RIL) were extensively or fully genotyped with 121 evenly spaced SNP markers. The SNP markers used in this work were the same as previously applied for creating RIL population (Li *et al.* 2006). This genotyping procedure resulted in the recognition of approximately 20 NILs homozygous for a single donor chromosome segment (fully genotyped). To obtain more lines with single donor segment, the extensively (or fully) genotyped BC_3-BC_5S_{10} lines were further backcrossed to N2 males for 1 or 2 generations and subsequently selfed using the similar protocol as described above. In that way, the length or/and the number of the introgressed segments was further reduced. The lines, which contained more than one introgression are not included in the presented NIL library. All strains are stored as cultures at 15 ºC and as frozen stocks at -80 ºC (Lewis & Fleming 1995).

Nematode culturing and behavioural assays

The nematodes were cultured and subjected to behavioural assays on 6 cm Petri dishes with the nematode growth medium, NGM, containing 2% agar, seeded with *Escherichia coli* OP50 in LB medium and incubated overnight at 37 ºC. For the purpose of the assays, the bacteria were distributed over the medium surface to create a lawn of approximately 3 cm diameter. The dishes were stored for four days at 12 ºC before

use. Prior to the assays, all lines were grown at 20 ºC under standard conditions for at least two weeks. Synchronized eggs (Emmons *et al.* 1979) in the numbers within the range of 40-300, were placed on the assay dishes and subsequently stored at 20 ºC in dark. After approximately 40 hours (nematodes in fourth larval stage, L4), clumping behaviour was assessed as the fraction of individuals, which were in contact with at least two other worms along at least 50% of their body length. (de Bono & Bargmann 1998).

Mapping procedure

The evaluation of clumping behaviour for the RIL mapping population and the parental lines was performed in three batches (23 or 24 randomly assigned lines per batch) with 2 replicate assay dishes per RIL/parental line. The detailed description of the genetic constitution of the used RILs as well as the information on the construction of the linkage map can be found in Li *et al.* (2006). The analysis of 19 NILs was carried out in a single batch using ten replicate assay dishes per NIL and N2 parental line.

Statistical and QTL analyses

All aggregation data was recorded as frequencies and, therefore, was transformed using the equation: $y' = \sin^{-1}\sqrt{y}$ (Xu 2002). QTL mapping with the RIL population was performed with the composite interval mapping procedure (Zeng 1994) in QTL Cartographer (Version 2.5) applying model 6 with a 5- and 10-cM window size and 10 background parameters. Since the window size showed little influence on the significance of the detected peaks, we present only the results of the model with 10-cM window size and 10 background parameters (chosen with the automatic selection option). The analyses were done on the lines' means of the residual effects after the correction for batch effect. The used significance threshold was a LOD score of 2.7, based on thresholds previously obtained by 10000 random permutations on life-history traits mapping data of the same RIL population (Churchill & Doerge 1994; Gutteling *et al.* 2006). In order to test for epistasis between the detected QTLs, we looked for pair-wise interactions using ANOVA with markers closest linked to the significant QTLs as random factors. Dunn-Šidak adjustment was implemented to correct for multiple testing. The contribution of each QTL and the identified interactions to the total variance was estimated with stepwise multiple regression with all detected QTLs (represented by the closest linked markers) and the significant interactions as independent variables. The detection of QTLs for the set of NILs was carried out by comparing the means of an individual NIL with the mean of N2 animals, using ANOVA. To correct for multiple testing, the significance level was

adjusted with Dunn-Šidak procedure. The analyses were performed in SPSS and SAS using PROC GLM procedure.

Discussion

We report the construction and properties of a genome-wide library consisting of 87 near-isogenic lines, a powerful resource to study complex traits in *C. elegans*. The subset of the created lines was used to search for novel loci associated with aggregation behaviour to illustrate the potential application of this resource. We believe that the presented NIL library will accelerate the discovery of natural polymorphisms and will lead to better understanding of the mechanisms behind observed phenotypic variation.

The choice of the parental strains used for the derivation of any mapping population, thus also NIL library, has a profound influence on the results of QTL mapping and fine-mapping. The genetic differences between the parental strains predetermine which QTLs can be detected and affect the power of genetic dissection. While the overall level of polymorphism is rather low among wild isolates of *C. elegans*, the genetic distance between N2 and CB4856 is relatively high. In fact, CB4856 was reported to show the highest genetic distance from N2 compared to other wild isolates (Koch *et al.* 2000; Denver *et al.* 2003). This divergence is also reflected by the profound differences in the traits such as ethanol responses (Davies *et al.* 2004), clumping behaviour (De Bono & Bargmann 1998), RNAi sensitivity (Tijsterman *et al.* 2002), formation of copulatory plug (Hodgkin & Doniach 1997) and many life-history traits (Gutteling *et al.* 2006). Also a high number of the single nucleotide polymorphisms (SNP), of which 121 were used for the determination of genetic constitution of the constructed NILs, indicates substantial genetic differences between these strains (Koch *et al.* 2000). The use of the N2 strain as the background for CB4856 introgressions is additionally justified by the common use of N2 in *C. elegans* research and the wealth of available genetic and genomic tools.

In principle, there are two main approaches of QTL mapping using NIL libraries. In the first one, a number of NILs is selected based on the location of QTLs previously identified with the use of another mapping population (e.g. RILs) and analysed to confirm and fine-map the putative QTLs. The second approach involves direct library-wide screens. The advantage of the first approach is that it might result in the initial detection of QTLs interacting with another loci, which might become undetectable when introgressed to a common background. These QTLs can be further analysed with the lines carrying combined donor segments constructed from multiple NILs. The second approach, in turn, allows detection of the QTLs of small effects due to the reduced background noise and does not require primary QTL analysis with

other mapping populations (Iakoubova *et al.* 2001).

While the examination of 121 SNP markers indicated no undesired CB4856 segments in the presented NILs (except WN19-3), the presence of additional short donor segments within a region flanked by two adjacent markers (or the regions between the ends of the chromosomes and the closest markers) cannot be entirely excluded. Genotyping using higher marker density would allow detection of the additional CB4856 segments and taking them into account in QTL analysis. Nevertheless, the current average distance between markers is 2.38 cM and the probability of the occurrence of even number of crossovers within such small genomic region is low. Consequently, undetected donor segments are expected to be rare.

We were able to detect three significant QTLs associated with clumping behaviour in *C. elegans* using RIL mapping population and CIM. The loci on chromosome *III* (agr3) and on chromosome *X* (agrX) explained approximately 13 and 5% of total variance, respectively and were confirmed by the analysis of the NILs. The NIL analysis did not provide sufficient evidence to support the presence of QTL on chromosome *IV* (agr4). While the analysis of the RIL mapping population showed enhancing effect of N2 alleles at agr3 on clumping behaviour, in NILs aggregation was enhanced by CB4856 alleles at this locus, what suggests that the effect of the allele at agr3 depends on genetic background. This pattern, cannot be, however, explained by epistatic effects between agr3 and any of two other detected loci (agr4 and agrX), as the pair-wise tests of interactions were insignificant.

The position of agrX, as identified by CIM and subsequent NIL analysis, coincides with *npr-1* (the closest marker, pkP6107), the only gene known to contribute to natural variation in clumping behaviour. *npr-1* encodes G protein-coupled receptor related to mammalian neuropeptide Y receptor and its two allelic forms differing at a single amino acid occur in wild isolates. The wild isolates having valine at the residue 215 of the *npr-1* gene are solitary, show low locomotory activity and fail to avoid high oxygen when food is present, while the isolates having phenylalanine aggregate together, move rapidly and avoid hyperoxia when food is abundant (de Bono *et al.* 2002; Gray *et al.* 2004; de Bono & Maricq 2005; Chang *et al.* 2006). The correspondence of our results with the well documented role of *npr-1* in regulation of clumping behaviour provides validation to our approach. In this study the strongest association of clumping behaviour was found for agr3 which was localized on the left arm of the chromosome *III*. Despite the relatively small size of the region containing agr3 determined with NIL analysis, it was difficult to identify candidate genes. None of the genes within this region has been reported to be related with behaviour phenotypes or to have an influence on the functioning of the nervous system. In addition, the reports on RNAi-induced phenotypes of predicted genes, did not provide sufficient information to select obvious candidates (WormBase;

http://www.wormbase.org/). Taking a less conservative approach, the searched region can be extended by including confidence interval area obtained with CIM analysis of the RIL population (which is not contradicting the results of NIL analysis). This interval contains *daf-7* (the closest marker, pkP3045), which encodes TGF-β-related protein in the dauer formation pathway involved in the development of the third larval stage, which is specialized to survive in stressful environments (Ren *et al.* 1996). There are several reasons to consider *daf-7* as an important candidate gene for regulation of clumping behaviour in natural populations. The mutants of *daf-7* were shown to aggregate in the presence of food (de Bono *et al.* 2002). The recent study of Chen and colleagues showed, that *daf-7* suppresses hyperoxia avoidance by inhibition of serotonin synthesis in ADF neurons (Chang *et al.* 2006). The relevance of these results is based on the reasoning, that aggregation behaviour is partly driven by hyperoxia avoidance. Local aggregation reduces the contact of the animals with oxygen, thereby preventing its deleterious metabolic and mutagenic effects (Chang *et al.* 2006). The same authors suggest also, that serotonin induction in ADF is a common stress-related response, since it applies to high temperature, bacterial pathogens and genes in the dauer formation pathway. Overall, at this stage, there is not sufficient evidence for functional involvement of any of genes within the agr3 region. More insight into the architecture of agr3 can be gained by analysis of sub-NILs and further investigation of candidate genes (also *daf-7*) using strategies such as complementation tests, positional cloning and functional analyses.

Currently, one of the greatest challenges of QTL research is the development of strategies and resources, which allow efficient dissection of the QTLs of small effects, enable to locate them with high accuracy and facilitate gene identification. The presented genomic library of near-isogenic lines is the first step to achieve these goals and significantly improve QTL detection process in *C. elegans*.

Acknowledgements

We thank the *Caenorhabditis* Genetics Center for providing the nematode strains N2 and CB4856. The work was supported by the TRIAS Programme (TRIpartite Approach to Soil system processes) of Delft Cluster, SKB (Foundation for development and transmission of soil knowledge) and NWO (the Netherlands Organisation for Scientific Research), as project 835.80.008.

References

Ayyadevara, S., Ayyadevara, R., Hou, S., Thaden, J.J. & Reis, R.J.S. (2001) Genetic mapping of quantitative trait loci governing longevity of *Caenorhabditis elegans* in recombinant-inbred progeny of a Bergerac-BO x RC301 interstrain cross. *Genetics*, **157**, 655-666.

Ayyadevara, S., Ayyadevara, R., Vertino, A., Galecki, A., Thaden, J.J. & Reis, R.J.S. (2003) Genetic loci modulating fitness and life span in *Caenorhabditis elegans*: Categorical trait interval mapping in CL2a X Bergerac-BO recombinant-inbred worms. *Genetics*, **163**, 557-570.

Chang, A.J., Chronis, N., Karow, D.S., Marletta, M.A. & Bargmann, C.I. (2006) A distributed chemosensory circuit for oxygen preference in *C. elegans*. *Plos Biology*, **4**, 1588-1602.

Churchill, G.A. & Doerge, R.W. (1994) Empirical threshold values for quantitative trait mapping. *Genetics*, **138**, 963-971.

Clark, R.M., Wagler, T.N., Quijada, P. & Doebley, J. (2006) A distant upstream enhancer at the maize domestication gene tb1 has pleiotropic effects on plant and inflorescent architecture. *Nature Genetics*, **38**, 594-597.

Clee, S.M., Yandell, B.S., Schueler, K.M., Rabaglia, M.E., Richards, O.C., Raines, S.M., Kabara, E.A., Klass, D.M., Mui, E.T.K., Stapleton, D.S., Gray-Keller, M.P., Young, M.B., Stoehr, J.P., Lan, H., Boronenkov, I., Raess, P.W., Flowers, M.T. & Attie, A.D. (2006) Positional cloning of Sorcs1, a type 2 diabetes quantitative trait locus. *Nature Genetics*, **38**, 688-693.

Consortium, T.C.e.S. (1998) Genome sequence of the nematode *C. elegans*: A platform for investigating biology. *Science*, **282**, 2012-2018.

Darvasi, A. & Soller, M. (1995) Advanced intercross lines, an experimental population for fine genetic mapping. *Genetics*, **141**, 1199-1207.

Davies, A.G., Bettinger, J.C., Thiele, T.R., Judy, M.E. & McIntire, S.L. (2004) Natural variation in the npr-1 gene modifies ethanol responses of wild strains of *C. elegans*. *Neuron*, **42**, 731-743.

Davis, R.C., Schadt, E.E., Smith, D.J., Hsieh, E.W.Y., Cervino, A.C.L., van Nas, A., Rosales, M., Doss, S., Meng, H., Allayee, H. & Lusis, A.J. (2005) A genome-wide set of congenic mouse strains derived from DBA/2J on a C57BL/6J background. *Genomics*, **86**, 259-270.

de Bono, M. & Bargmann, C.I. (1998) Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in *C. elegans*. *Cell*, **94**, 679.

de Bono, M., Tobin, D.M., Davis, M.W., Avery, L. & Bargmann, C.I. (2002) Social feeding in *Caenorhabditis elegans* is induced by neurons that detect aversive stimuli. *Nature*, **419**, 899-903.

de Bono, M. & Maricq, A.V. (2005) Neuronal substrates of complex behaviours in *C. elegans*. *Annual Review of Neuroscience*, **28**, 451-501.

Denver, D.R., Morris, K. & Thomas, W.K. (2003) Phylogenetics in *Caenorhabditis elegans:* An analysis of divergence and outcrossing. *Molecular Biology and Evolution*, **20**, 393-400.

Eduardo, I., Arus, P. & Monforte, A.J. (2005) Development of a genomic library of near isogenic lines (NILs) in melon (*Cucumis melo L.*) from the exotic accession PI161375. *Theoretical and Applied Genetics*, **112**, 139-148.

Emmons, S.W., Klass, M.R. & Hirsh, D. (1979) Analysis of the constancy of DNA sequences during development and evolution of the nematode *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences, USA*, **76**, 1333-1337.

Eshed, Y. & Zamir, D. (1995) An introgression line population of *Lycopersicon pennellii* in the cultivated tomato enables the identification and fine mapping of yield-associated QTL. *Genetics*, **141**, 1147-1162.

Flint, J. & Mott, R. (2001) Finding the molecular basis of quantitative traits: Successes and pitfalls. *Nature*

Reviews Genetics, **2**, 437-445.

Flint, J., Valdar, W., Shifman, S. & Mott, R. (2005) Strategies for mapping and cloning quantitative trait genes in rodents. *Nature Reviews Genetics*, **6**, 271-286.

Fridman, E., Liu, Y.S., Carmel-Goren, L., Gur, A., Shoresh, M., Pleban, T., Eshed, Y. & Zamir, D. (2002) Two tightly linked QTLs modify tomato sugar content via different physiological pathways. *Molecular Genetics and Genomics*, **266**, 821-826.

Gami, M.S. & Wolkow, C.A. (2006) Studies of *Caenorhabditis elegans* DAF-2/insulin signaling reveal targets for pharmacological manipulation of lifespan. *Aging Cell*, **5**, 31-37.

Giles, A.C., Rose, J.K. & Rankin, C.H. (2006) Investigations of learning and memory in *Caenorhabditis elegans*. *Neurobiology of C. Elegans*, pp. 37.

Gotz, J., Streffer, J.R., David, D., Schild, A., Hoerndli, F., Pennanen, L., Kurosinski, P. & Chen, F. (2004) Transgenic animal models of Alzheimer's disease and related disorders: Histopathology, behaviour and therapy. *Molecular Psychiatry*, **9**, 664-683.

Gray, J.M., Karow, D.S., Lu, H., Chang, A.J., Chang, J.S., Ellis, R.E., Marietta, M.A. & Bargmann, C.I. (2004) Oxygen sensation and social feeding mediated by a *C. elegans* guanylate cyclase homologue. *Nature*, **430**, 317.

Gutteling, E.W., Riksen, J.A.G., Bakker, J. & Kammenga, J.E. (2006) Mapping phenotypic plasticity and genotype-environment interactions affecting life-history traits in *Caenorhabditis elegans*. *Heredity*, in press

Hodgkin, J. & Doniach, T. (1997) Natural variation and copulatory plug formation in *Caenorhabditis elegans*. *Genetics*, **146**, 149-164.

Iakoubova, O.A., Olsson, C.L., Dains, K.M., Ross, D.A., Andalibi, A., Lau, K., Choi, J., Kalcheva, I., Cunanan, M., Louie, L., Nimon, V., Machrus, M., Bentley, L.G., Beauheim, C., Silvey, S., Cavalcoli, J., Lusis, A.J. & West, D.B. (2001) Genome-tagged mice (GTM): Two sets of genome-wide congenic strains. *Genomics*, **74**, 89-104.

Knight, C.G., Azevedo, R.B.R. & Leroi, A.M. (2001) Testing life-history pleiotropy in *Caenorhabditis elegans*. *Evolution*, **55**, 1795-1804.

Koch, R., van Luenen, H., van der Horst, M., Thijssen, K.L. & Plasterk, R.H.A. (2000) Single nucleotide polymorphisms in wild isolates of *Caenorhabditis elegans*. *Genome Research*, **10**, 1690-1696.

Korstanje, R. & Paigen, B. (2002) From QTL to gene: the harvest begins. *Nature Genetics*, **31**, 235-236.

Kurz, C.L. & Ewbank, J.J. (2003) *Caenorhabditis elegans:* An emerging genetic model for the study of innate immunity. *Nature Reviews Genetics*, **4**, 380-390.

Lewis, J.A. & Fleming, J.T. (1995) Basic culture methods. *Caenorhabditis elegens: modern biological analysis of an organism* (eds H.F. Epstein & D.C. Shakes), pp. 3-29. Academic Press, Inc., San Diego, California.

Li, Y., Alda-Alvarez, O., Gutteling, E.W., Tijsterman, M., Fu, J., Riksen, J.A.G., Hazendonk, E., Prins, P., Plasterk, R.H.A., Jansen, R.C., Breitling, R. & Kammenga, J.E. (2006) Mapping determinants of gene expression plasticity by genetical genomics in *C. elegans*. *Plos Genetics, **12**, e222*.

Li, Z.K., Fu, B.Y., Gao, Y.M., Xu, J.L., Ali, J., Lafitte, H.R., Jiang, Y.Z., Rey, J.D., Vijayakumar, C.H.M., Maghirang, R., Zheng, T.Q. & Zhu, L.H. (2005) Genome-wide introgression lines and their use in genetic and molecular dissection of complex phenotypes in rice (*Oryza sativa L.*). *Plant Molecular Biology*, **59**, 33-52.

Mackay, T.F.C. (2001) Quantitative trait loci in *Drosophila*. *Nature Reviews Genetics*, **2**, 11-20.

Paran, I. & Zamir, D. (2003) Quantitative traits in plants: beyond the QTL. *Trends in Genetics*, **19**, 303-306.

Pestsova, E.G., Borner, A. & Roder, M.S. (2006) Development and QTL assessment of *Triticum aestivum-Aegilops tauschii* introgression lines. *Theoretical and Applied Genetics*, **112**, 634-647.

Ren, P.F., Lim, C.S., Johnsen, R., Albert, P.S., Pilgrim, D. & Riddle, D.L. (1996) Control of *C. elegans* larval development by neuronal expression of a TGF-beta homolog. *Science*, **274**, 1389-1391.

Shook, D.R., Brooks, A. & Johnson, T.E. (1996) Mapping quantitative trait loci affecting life history traits in the nematode *Caenorhabditis elegans*. *Genetics*, **142**, 801-817.

Shook, D.R. & Johnson, T.E. (1999a) Quantitative trait loci affecting survival and fertility-related traits in *Caenorhabditis elegans* show genotype-environment interactions, pleiotropy and epistasis. *Genetics*, **153**, 1233-1243.

Shook, D.R. & Johnson, T.E. (1999b) Quantitative trait loci affecting survival and fertility- related traits in *Caenorhabditis elegans* show genotype- environment interactions, pleiotropy and epistasis. *Genetics*, **153**, 1233-1243.

Swan, K.A., Curtis, D.E., McKusick, K.B., Voinov, A.V., Mapa, F.A. & Cancilla, M.R. (2002) High-throughput gene mapping in *Caenorhabditis elegans*. *Genome Research*, **12**, 1100-1105.

Thomson, M.J., Edwards, J.D., Septiningsih, E.M., Harrington, S.E. & McCouch, S.R. (2006) Substitution mapping of dth1.1, a flowering-time quantitative trait locus (QTL) associated with transgressive variation in rice, reveals multiple sub-QTL. *Genetics*, **172**, 2501-2514.

Tijsterman, M., Okihara, K.L., Thijssen, K. & Plasterk, R.H.A. (2002) PPW-1, a PAZ/PIWI protein required for efficient germline RNAi, is defective in a natural isolate of *C. elegans*. *Current Biology*, **12**, 1535-1540.

Valdar, W., Solberg, L.C., Gauguier, D., Burnett, S., Klenerman, P., O Cookson, W., Taylor, M.S., Rawlins, J.N.P., Mott, R. & Flint, J. (2006) Genome-wide genetic association of complex traits in heterogeneous stock mice. *Nature Genetics*, **38**, 879-887.

Van Swinderen, B., Shook, D.R., Ebert, R.H., Cherkasova, V.A., Johnson, T.E., Reis, R.J.S. & Crowder, C.M. (1997) Quantitative trait loci controlling halothane sensitivity in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences, USA*, **94**, 8232-8237.

Xu, S. (2002) QTL analysis in plants. *Quantitative Trait Loci: methods and protocols* (eds N.J. Camp & C. A.), pp. 283-310. Humana Press, Totowa, New Jersey.

Zeng, Z.B. (1994) Precision mapping of Quantitative Trait Loci. *Genetics*, **136**, 1457-1468.

Appendix 1

Positions of 121 SNP markers used in this study.

chromosome	marker	position	
		bp	cM
I	pkP1050	168807	-18.2603
	pkP1101	992188	-17.2825
	pkP1103	1884415	-11.959
	pkP1052	2818973	-6.1004
	egPE107	3502476	-3.5488
	egPF101	4338254	-1.4887
	pkP1054	4845515	-0.6162
	egPH102	5893622	0.4597
	pkP1057	6359867	0.9366
	pkP1116	7589863	2.1576
	egPK103	7894081	2.4087
	pkP1059	8654360	2.9456
	pkP1122	9569914	3.7959
	egPN104	10259909	4.7801
	egPO105	11085295	6.0193
	pkP1068	11760182	7.5226
	pkP1070	12614280	13.8659
	egPR106	13280531	17.4648
	pkP1071	14162948	22.9129
	pkP1072	14958660	28.9192
II	pkP2101	176721	-17.378
	pkP2135	1270097	-15.4084
	pkP2136	1683953	-14.914
	pkP2114	2755074	-10.0773
	egPE201	3403575	-5.9476
	egPF202	4147051	-5.4083
	egPG203	4800868	-3.5209
	pkP2147	5761743	-0.9662
	pkP2106	6427387	-0.316
	egPJ204	7257517	0.4409
	pkP2151	8195651	0.7867
	egPL205	8748358	0.8759
	pkP2109	9401070	1.2287
	pkP2124	10414073	2.4889
	pkP2110	11180836	3.2851
	egPP206	11752128	4.0335
	egPQ207	12630696	10.8343
	pkP2116	13235559	15.8706
	egPS208	13980541	22.0573
	egPT209	14758293	29.1025

III	egPA301	360894	-27.0345
	pkP3045	840469	-25.8751
	egPC302	1893290	-18.0248
	egPD303	2459092	-13.6165
	pkP3057	3068098	-9.3329
	pkP3096	3923438	-4.3164
	egPG306	4517529	-3.2032
	pkP3047	5431252	-1.7369
	egPI313	5925983	-1.4086
	pkP3100	6847169	-0.9176
	egPK314	7320107	-0.7451
	egPL308	7998164	-0.4552
	pkP3049	8318553	-0.3121
	egPN309	9308858	0.3746
	egPO310	10027496	1.4094
	pkP3059	10613119	4.3513
	pkP3060	11341120	8.6849
	pkP3075	12301725	14.9161
	pkP3113	12739834	18.4884
	egPT312	13220073	20.8962
IV	egPA410	151889	-27.2932
	pkP4051	1381409	-19.9736
	egPC402	2288742	-8.5343
	egPD403	3067374	-4.7814
	egPE404	3920366	-1.7108
	pkP4071	4991858	1.4532
	egPG405	5819735	2.4204
	pkP4075	6599685	3.2114
	egPI406	7405743	3.3697
	pkP4039	8397264	3.7498
	pkP4080	9102404	4.0579
	dbP3	10122930	4.5536
	dbP6	10909560	4.7525
	pkP4043	11668242	5.1651
	pkP4047	12748880	6.238
	pkP4048	13667267	10.2031
	pkP4095	14566567	12.1345
	egPR407	15178091	13.0337
	egPS408	16371991	15.0474
	egPT409	17084259	16.2268
V	egPA501	590471	-20.0903
	pkP5076	1773465	-17.4243
	egPC502	2878209	-12.0977
	egPD503	3606323	-8.8643
	egPE504	4550758	-4.953
	egPF505	5814403	-0.67
	pkP5113	6612376	0.131
	egPH506	7690531	0.8527

	pkP5116	8567608	1.4605
	egPI507	9405441	2.0011
	pkP5064	10368660	2.5149
	pkP5065	10912994	2.8247
	pkP5067	11796050	3.3745
	egPM508	13033974	4.8313
	pkP5125	13951861	5.7659
	pkP5071	15158759	7.4648
	egPP509	16008404	9.299
	pkP5073	17377158	12.3353
	pkP5082	18574593	16.381
	egPS510	19525561	21.6572
	pkP5075	20758352	25.1471
X	pkP6140	696607	-19.3558
	pkP6142	1462371	-17.8928
	pkP6118	2389438	-15.1501
	pkP6120	3328811	-10.5284
	pkP6105	4161495	-8.1624
	pkP6107	5010049	-6.1517
	pkP6151	5770179	-4.2898
	pkP6155	7067019	-1.9388
	pkP6110	7982354	-0.937
	pkP6158	8691677	0.4135
	egPK601	9330879	1.1075
	pkP6128	10323428	1.8242
	pkP6114	11142277	3.0298
	pkP6125	12208257	6.8455
	pkP6131	12891891	8.9333
	pkP6163	13893785	14.4125
	egPQ603	14600585	16.6104
	egPR604	15620481	22.9098
	pkP6169	16366136	23.8963
	pkP6171	17614442	24.3623

Chapter 6

General discussion and concluding remarks

Agnieszka Doroszuk

The main aims of this thesis were to obtain a broad picture of the impact of long-term stress on natural populations by implementing an ecological and evolutionary genetics perspective and to provide the tools for the analysis of the genetic basis of stress response and resistance. The analysis of *A. nanus* populations inhabiting the Bovenbuurt experimental field led to several interesting observations and conclusions. First, the results showed that population functions (secondary production and biomass turnover rate) might be more sensitive to multiple stressors than structural population parameters like biomass or population density. Therefore, focusing only on structural aspects of population or community response to stressors (although easier to perform) might result in underestimation of the adverse effects. Within the same chapter, we also report that the changes in body growth rate (which translate to the length of developmental time) were the most important drivers of the effects on the level of populations functions. This fact signifies the importance of the effects of life-history traits on population functions. Second, the evidence for adaptive divergence within the population of *A. nanus* exposed for 20-years to different stress regimes implies that asexually reproducing populations are capable of rapid adaptive evolution under field conditions and that structure and distribution of asexual populations can be shaped by local adaptation events. These results provide several additional insights. Namely, they indicated that the stress-adapted populations suffered no apparent reduction in biomass under stressful conditions compared with the control populations, while there was a substantial adverse effect on functional population parameters. In other words, adaptation enabled the persistence of the populations, however, it was less efficient in rescuing the initial populations' functions. It should be realized that the values of the population parameters were influenced by the adaptive changes in life-history traits and, consequently, the effects of the same stressors on a non-adapted population shortly after stress application could have been more severe. Thus, it can be concluded that evolutionary processes operate on ecologically relevant time-scales and are potentially important contributors to ecologically relevant population responses. Third, the divergence of genetic variance-covariance matrices within the population of *A. nanus* within 20 years implies, that genetic relationships among traits can evolve rapidly in nature. The most important implication of this finding is that it questions the possibility of predicting evolutionary responses and retrospective selection analyses, which assume stability of **G** matrix. Moreover, it can be concluded that selective pressure imposed by stressors and adaptation process shape genetic relationships among multiple characters. These alterations of the structure of **G** matrix not only affect the direction of evolution, but through the changes in genetic trade-offs might also influence population's structure and functions.

Sex and stress

Sexual reproduction requires investment of extra time and energy and has a disadvantage of disrupting favourable gene combinations. For this reason, its persistence and widespread occurrence have been a major puzzle for evolutionary biologists (Williams 1975; Maynard Smith 1978; Bell 1982; Stearns 1987). Modern theories explain the advantage of sex with various adaptive effects of recombination (West *et al.* 1999). One of these explanations was proposed more than 100 years ago by Weismann (1889), who suggested that recombination enhances the efficiency of selection and accelerates the adaptive process to a novel (stressful) environment by increasing genetic variation. This view was later supported by many experimental data (e.g. Birdsell & Wills 1996; Colegrave 2002; Goddard *et al.* 2005). One of the consequences of this reasoning is that asexual populations are expected to have a lower adaptive potential and to be more prone to local extinction. In this context, our findings are intriguing, as *A. nanus* appeared to experience local adaptation rather than local extinction. Also with the comparison to other community members, *A. nanus* appeared to perform very well, with its abundance being approximately 7% of the total nematode community under control conditions and reaching approximately 20% under the most stressful conditions (unpublished data). Despite the intuitive contradiction with the described predictions of low adaptability, the observations of this kind are not uncommon. Already in the seventies of the twentieth century it was noticed that asexual taxa are usually distributed over broader latitudes than their sexual relatives and that parthenogens often occupy more disturbed habitats (Weider 1993). The wide distribution of parthenogenetic species became a subject of debate. Lynch (1984) proposed that the success of asexual species in a range of environments could be attributed to their general purpose genotypes. This hypothesis was based on the older idea explaining geographic distribution of the weedy plants (Baker 1965) and on the reasoning that selection operates differently on fitness of asexual and sexual organisms (Lynch 1984). According to this concept, long-term clonal selection will favour low variance in fitness across a variety of environmental conditions and will promote the evolution of genotypes with broad tolerance ranges. One of the consequences of this idea is that local adaptation is predicted to play rather a minor role in distribution of asexual species. Empirical studies do not provide, however, a strong support for this theory and the tests of asexual populations and their sexual equivalents for tolerance across a range of stressful environments show inconsistent results (e.g. Weider 1993; Álvarez *et al.* 2005). On the other hand, adaptive capabilities of parthenogenetic species under field conditions are poorly documented (in spite of relatively high number of studies reporting adaptation in laboratory experiments). Our study provides the evidence for adaptation to local divergent stress regimes within 20 years and strongly suggests that the role of local adaptation in shaping the structure and distribution of asexual populations is underestimated. These conclusions evoke

a number of questions: How common is local adaptation in parthenogenetic species? If it is common, how do we need to modify the idea of general purpose genotype? What are the mechanisms behind adaptability of *A. nanus*? How common are these mechanisms among asexuals?

The range of adaptive phenotypic solutions to environmental problems is determined by genetic relationships between traits summarized by **G** matrix. As already mentioned, we detected divergence of **G** structure in life-history traits of *A. nanus* within 20 years. These results not only show that genetic architecture can evolve fast in nature, but also, together with several other studies (Deng & Lynch 1996; Pfrender & Lynch 2000) suggest that lower stability of **G** matrices might be a general property of asexual species. Consequently, in these species new phenotypic solutions and possibilities for adaptations might arise within relatively short temporal and spatial scales through the changes of genetic variance-covariance structure.

Clones and risk assessment

The problems discussed in the previous section have also their practical aspect related to risk assessment strategies of environmental contaminants. Clonal organisms and inbred strains are attractive subjects for laboratory toxicity tests because of the ease of culturing, possibility of collection of large data sets within short time-spans and little data variability. For these reasons, some clonal organisms such as *Daphnia magna* and *Folsomia candida* became popular subjects of toxicity tests (Adema 1978; Heugens *et al.* 2001; Fountain & Hopkin 2005). It remains, however, uncertain whether the results of tests on clonal organisms are representative for sexual species and what they can tell us about long-term risk for natural populations. Some studies showed that the results of toxicity tests on one species cannot be directly compared with the values for other species (Stark *et al.* 2004) and it is likely that the discrepancies between the species with different reproductive modes are even greater.

When we consider the matter from the evolutionary perspective and assume that the hypothesis of general purpose genotype is in principle valid then we need also to realize its implications for the interpretation of the toxicity experiments. According to this idea, asexual species have broader ranges of tolerance. Consequently, we might expect that risk assessment performed with these species would systematically underestimate the risk for most groups of sexual organisms. On the other hand, it would imply that the tests on one isolate of an asexual species are indicative for other isolates of the same species. If local adaptation was a more common manner of coping with divergent environments, we would expect more variability in stress resistance within a single clonal species. Likewise, we would not expect any systematic underestimation of toxic effects when extrapolating to other species.

Although the problem of extrapolation of the results of toxicity tests performed on genetically uniform clones to variable populations under field conditions has received some attention (e.g. Donald *et al.* 1989; Snell 1999), the role of genetic variation in shaping stress responses in asexuals has not been fully acknowledged. The evidence presented in this thesis strongly supports the idea that genetic factors might commonly influence stress response of asexuals in nature. Therefore, they should not be neglected while designing toxicity tests. Overall, due to the high specificity of this group with its characteristic life-history strategies and 'evolutionary properties', toxicity tests using clonal species might not sufficiently represent the effects on sexual species.

QTL approach and stress

Understanding the mechanisms of physiological and behavioural responses to stress, which might lead to the changes on higher organisation levels, requires incorporation of genetic analysis. While various approaches might be taken to identify the genes regulating responses to stress, QTL mapping provides insight into the subset of these genes contributing to natural variation. The information obtained with QTL studies is not restricted to the detection of natural variants of genes involved in stress response but it also provides insights into other relevant aspects of stress biology. Firstly, QTL analysis of stress-related traits might help to explain ecological observations such as species distribution or abundance. For example, Norry *et al.* (2004) provided an insight into genetic regulation of the ecologically relevant trait of knockout resistance to high temperature in *Drosophila melanogaster*. This trait is common in natural populations of small insects and describes a reaction of temporal incapacitation of individuals by heat. Secondly, QTL approach offers possibility to investigate genetic bases of trade-offs. Such application of QTL mapping is of a special importance in stress-related research because trade-offs are central for the main conceptual and analytic frameworks in stress response analysis (Sibly & Calow 1989; Sibly 1996; Maltby 1999). QTL studies can provide genetic explanation for the observed relationships among traits such as positive correlations between resistance traits for different stressors or negative relation between stress resistance and reproductive output. Since pleiotropy is one of the main causes of genetic correlations and trade-offs (Lynch & Walsh 1998), the investigation of pleiotropic effects of QTLs or genes involved in stress responses or resistance is one of the major focuses of QTL mapping. Thirdly, in agriculture, resistance of various crops to abiotic stressors such as drought, salinity or extreme temperatures is very important for insuring high production and income. There is, therefore, considerable attention for QTL-based approaches investigating genetic determination of stress resistance (e.g. Ren *et al.* 2005; Li *et al.* 2006; Yue *et al.* 2006). These studies are often directed towards practical goals such as finding natural gene

variants which are able to equip the susceptible but productive crops in desired stress resistance.

Despite its broad potential utility and growing popularity, the QTL approach has been criticized for relatively low efficiency of QTL detection and for low success rate in gene identification (Nadeau & Frankel 2000). This criticism was considered by many researchers to come too early, as it was based on only approximately 10 years of QTL studies (Korstanje & Paigen 2002; Abiola *et al.* 2003). However, it needs to be acknowledged that new developments in the area of research resources and analysis methods can substantially improve the efficiency and accuracy of QTL mapping. The genome-wide library of near-isogenic lines (NIL) of *C. elegans* is a promising resource, which will make the way from QTL to gene identification shorter. It is important to realize that quantitative genetic approaches are still marginal within *C. elegans* research and that the NIL library presented in this thesis is one of the first attempts to make QTL analysis in *C. elegans* more accessible and efficient. Combining the 'main-stream' *C. elegans* research methods with those of QTL approach will lead to a better understanding of the mechanisms behind observed phenotypic variation. For example, validation of the functions of the candidate genes identified with the QTL approach will benefit from the information on various pathways and the advanced methods of genetic manipulation. On the other hand, QTL studies are likely to indicate genes within the pathways showing natural polymorphism, which is often relevant for the performance of natural populations.

Future directions

Studying stress is multidisciplinary by definition and concerns various levels of biological organisation. Understanding of the linkages among these levels seems to be currently the greatest challenge of stress-related research. Although it is unrealistic to imagine a researchers to conduct investigations on cell- as well as ecosystem-level, there are several ways that offer possibility of integrating this multi-level and multidisciplinary information. For instance, one of the possible strategies for an integrative approach could involve analysis of the same experimental system by differently specialized but cooperating groups of researchers. The advantage of this approach is that the results collected in that manner would supplement each other and lead to insights on the links among the various levels.

Another aspect of stress-related research is that it remains dominated by test-based approaches complicating generalization and extrapolation of the observed response patterns. In contrast, research based on the hypotheses derived from the theories within major disciplines such as ecology or evolutionary genetics has a potential not only to result in more general interpretation of the experimental

outcomes, but would also contribute to advances in fundamental knowledge within these disciplines. For example, the studies of the evolution of resistance can provide valuable information from the perspective of long-term stress response and, at the same time, allow to investigate adaptation process from the perspective of evolutionary theory. This example is presented to emphasize the broad aspect of stress research. Overall, stress-related research would benefit from the integration of all, ecological, microevolutionary and genetic aspects.

New developments and application of novel techniques are relevant for the advances of any scientific field. Recent achievements and rapid development in the field of molecular genetics and genomics offer new insights into mechanistic explanations of stress responses and open new ways for modern risk assessment strategies. Combining the analyses of expressed genotypes and the performance of the equivalent phenotypes allows studying the mechanisms and functional stress responses in an integrative way (Dicke *et al.* 2004; Snape *et al.* 2004).

What concerns more specific topics touched upon in this thesis, I would like to bring into consideration several understudied problems, which deserve more attention in future research:

- Although the effects of mixed toxicity have been relatively intensively studied under laboratory conditions, it is important to learn more about the joint effects of stressors on natural populations. The results reported in chapter 2 are one of the first attempts in this area. Because of potential interactive and indirect effects influencing overall response of populations to multiple stressors, research systems and experimental setups should be chosen carefully. The preferred experimental designs (e.g. factorial designs) should enable relatively simple interpretation of results and allow the identification of causal factors.

- The drivers of the changes in population functional parameters need further investigation under field conditions and should be complemented by laboratory studies. Among these drivers, life-history traits such as length of juvenile period require a broader analysis of their responses to multiple stressors.

- In order to get more insight into the adaptive changes reported in chapter 3, it should be determined whether the nematodes became adapted to both stressors independently or the resistance to one stressor appeared as a 'by-product' of the resistance to the other one. Additionally, the mechanisms underlying these adaptations could be resolved. New insights into the mechanisms of multiple adaptations will help to resolve whether the exposure to multiple stressors

reduces the likelihood of the development of stress resistance, as predicted by population genetic studies (Klerks 1999).

- As mentioned already in one of the previous sections of General Discussion, the findings presented in chapter 3 raise the questions about the source of genetic variance in the populations of *A. nanus* and other asexual species and their influence on adaptability. More extensive analysis of local adaptation events in asexual species might improve our understanding of evolutionary dynamics in asexuals with the implications for explaining the wide-spread occurrence of sexual reproduction.

- The main challenge in **G** matrix research is to find the relationships between evolutionary processes and the patterns of **G** matrix evolution. Therefore, while investigating **G** it is important to recognize the approaches and experimental designs maximizing the returned information. Following the line of the presented research (chapter 5), one of the topics requiring exploration involves possible differences in the way **G** matrices of sexual and asexual species respond to different evolutionary circumstances.

- The knowledge of the genetic and molecular bases underlying genetic variances and covariances is necessary to understand evolution of complex traits. Application of QTL mapping methods offers possibility to study them. The future research should, therefore, benefit from integrating these two, currently separated fields of quantitative genetics.

- Clumping behaviour in *C. elegans* is interpreted as a form of stress response. It would be interesting to demonstrate an adaptive value of this behaviour and investigate whether the natural polymorphism in the underlying gene(s) can be related to some environmental factors divergent between the sites of the strains' origin.

Main implications for environmental risk assessment

The main purpose of environmental risk assessment (ERA) is to provide information, which can be used to make decisions serving the protection of populations and ecosystems. The results of this thesis brought up several issues and arguments related to the latest discussion around improvements of ERA (Breitholtz *et al.* 2006; Walker 2006). As argued already in one of the previous section of General Discussion, the

extensive use of asexual organisms in tests is likely not to be the best choice despite their low-cost-related advantages. The presented results support also the view of some authors, who recommended to focus on population-level data, so that indirect effects and other ecological mechanisms are more acknowledged (Bechmann 1994; Calow 1996; Forbes & Calow 1999). At the same time, I would like to emphasize the need of testing individual life-history parameters, which are the main determinants of population-level responses. Interactive effects, such as the interactions of multiple stressors or ecological interactions among populations, contribute to population-level responses and the growing evidence of their relevance (chapter 2 this thesis, Preston 2002; Sih *et al.* 2004) should be reflected in the approaches taken in risk assessment. Adaptation and the changes in genetic properties of populations (as reported in chapter 3 and 4) are important effects of long-term stress. Since the standard toxicity tests commonly overlook these effects, ERA should also adopt long-term testing strategies, where genetic factors are examined and microevolutionary processes are acknowledged.

It is important to realize that the construction of an ideal toxicity test is impossible because the relations among the relevant properties of any toxicity test (cost, ecological relevance, reproducibility and sensitivity) have often the character of trade-offs (Breitholtz *et al.* 2006). However, it remains the responsibility of scientific community to challenge the existing concepts, indicate their possible caveats and pursue the practical approaches, which are grounded in scientific knowledge.

References

Abiola, O., Angel, J.M., Avner, P., Bachmanov, A.A., Belknap, J.K., Bennett, B., Blankenhorn, E.P., Blizard, D.A., Bolivar, V., Brockmann, G.A., Buck, K.J., Bureau, J.F., Casley, W.L., Chesler, E.J., Cheverud, J.M., Churchill, G.A., Cook, M., Crabbe, J.C., Crusio, W.E., Darvasi, A., de Haan, G., Demant, P., Doerge, R.W., Elliott, R.W., Farber, C.R., Flaherty, L., Flint, J., Gershenfeld, H., Gu, J., Gu, W.K., Himmelbauer, H., Hitzemann, R., Hsu, H.C., Hunter, K., Iraqi, F.A., Jansen, R.C., Johnson, T.E., Jones, B.C., Kempermann, G., Lammert, F., Lu, L., Manly, K.F., Matthews, D.B., Medrano, J.F., Mehrabian, M., Mittleman, G., Mock, B.A., Mogil, J.S., Montagutelli, X., Morahan, G., Mountz, J.D., Nagase, H., Nowakowski, R.S., O'Hara, B.R., Osadchuk, A.V., Paigen, B., Palmer, A.A., Peirce, J.L., Pomp, D., Rosemann, M., Rosen, G.D., Schalkwyk, L.C., Seltzer, Z., Settle, S., Shimomura, K., Shou, S.M., Sikela, J.M., Siracusa, L.D., Spearow, J.L., Teuscher, C., Threadgill, D.W., Toth, L.A., Toye, A.A., Vadasz, C., Van Zant, G., Wakeland, E., Williams, R.W., Zhang, H.G. & Zou, F. (2003) The nature and identification of quantitative trait loci: a community's view. *Nature Reviews Genetics*, **4**, 911-916.

Adema, D.M.M. (1978) *Daphnia magna* as a test animal in acute and chronic toxicity tests. *Hydrobiologia*, **V59**, 125-134.

Álvarez, O.A., Jager, T., Kooijman, S. & Kammenga, J.E. (2005) Responses to stress of *Caenorhabditis elegans* populations with different reproductive strategies. (vol 19, pg 656, 2005). *Functional Ecology*, **19**, 897-897.

Baker, H.G. (1965) Characteristics and and modes of origin of weeds. *Genetics of colonizing species* (eds H.G. Baker & G.L. Stebbins), pp. 137-172. Academic Press, New York.

Bechmann, R.K. (1994) Use of life-tables and LC50 tests to evaluate chronic and acute toxicity effects of copper on the marine copepod *Tisbe furcata* (Baird). *Environmental Toxicology and Chemistry*, **13**, 1509-1517.

Bell, G. (1982) *The masterpiece of nature: the evolution and genetics of sexuality* University of California Press, Berkeley.

Birdsell, J. & Wills, C. (1996) Significant competitive advantage conferred by meiosis and syngamy in the yeast *Saccharomyces cerevisiae*. *PNAS*, **93**, 908-912.

Breitholtz, M., Ruden, C., Hansson, S.O. & Bengtsson, B.E. (2006) Ten challenges for improved ecotoxicological testing in environmental risk assessment. *Ecotoxicology and Environmental Safety*, **63**, 324-335.

Calow, P. (1996) Ecology in ecotoxicology: some possible 'rules of thumb'. *ECOtoxicoloxy: ecological dimensions* (eds D.J. Baird, L. Maltby, P.W. Greig-Smith & P.E.T. Douben), pp. 5-12. Chapman and Hall, SETAC Press Series Cambridge.

Colegrave, N. (2002) Sex releases the speed limit on evolution. *Nature*, **420**, 664-666.

Deng, H.W. & Lynch, M. (1996) Change of genetic architecture in response to sex. *Genetics*, **143**, 203-212.

Dicke, M., van Loon, J.J.A. & de Jong, P.W. (2004) Ecogenomics benefits community ecology. *Science*, **305**, 618-619.

Donald, J.B., Ian, B., Mairead, B., Peter, C. & Amadeu, M.V.M.S. (1989) The *Daphnia* bioassay: a critique. *Hydrobiologia*, **188-189**, 403-406.

Forbes, V.E. & Calow, P. (1999) Is the per capita rate of increase a good measure of population-level effects in ecotoxicology? *Environmental Toxicology and Chemistry*, **18**, 1544-1556.

Fountain, M.T. & Hopkin, S.P. (2005) *Folsomia candida* (Collembola): A "standard" soil arthropod. *Annual Review of Entomology*, **50**, 201-222.

Goddard, M.R., Charles, H., Godfray, J. & Burt, A. (2005) Sex increases the efficacy of natural selection in

experimental yeast populations. *Nature*, **434**, 636-640.

Heugens, E.H.W., Hendriks, A.J., Dekker, T., van Straalen, N.M. & Admiraal, W. (2001) A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. *Critical Reviews in Toxicology*, **31**, 247-284.

Klerks, P.L. (1999) The influence of contamination complexity on adaptation to environmental contaminants. *Genetics and ecotoxicology* (ed V.E. Forbes), pp. 103-122. Taylor & Francis, Philadelphia.

Korstanje, R. & Paigen, B. (2002) From QTL to gene: the harvest begins. *Nature Genetics*, **31**, 235-236.

Li, J.Z., Huang, X.Q., Heinrichs, F., Ganal, M.W. & Roder, M.S. (2006) Analysis of QTLs for yield components, agronomic traits, and disease resistance in an advanced backcross population of spring barley. *Genome*, **49**, 454-466.

Lynch, M. (1984) Destabilizing hybridization, general-purpose genotypes and geographic parthenogenesis. *Quarterly Review of Biology*, **59**, 257-290.

Lynch, M. & Walsh, J.B. (1998) *Genetics and analysis of Quantitative Traits* Sinauer Associates, Sunderland, MA.

Maltby, L. (1999) Studying stress: The importance of organism-level responses. *Ecological Applications*, **9**, 431-440.

Maynard Smith, J. (1978) *The evolution of sex* Cambridge University Press, Cambridge.

Nadeau, J.H. & Frankel, W.N. (2000) The roads from phenotypic variation to gene discovery: mutagenesis versus QTLs. *Nature Genetics*, **25**, 381-384.

Norry, F.M., Dahlgaard, J. & Loeschcke, V. (2004) Quantitative trait loci affecting knockdown resistance to high temperature in *Drosophila melanogaster*. *Molecular Ecology*, **13**, 3585-3594.

Pfrender, M.E. & Lynch, M. (2000) Quantitative genetic variation in *Daphnia:* Temporal changes in genetic architecture. *Evolution*, **54**, 1502-1509.

Preston, B.L. (2002) Indirect effects in aquatic ecotoxicology: Implications for ecological risk assessment. *Environmental Management*, **29**, 311-323.

Ren, Z.H., Gao, J.P., Li, L.G., Cai, X.L., Huang, W., Chao, D.Y., Zhu, M.Z., Wang, Z.Y., Luan, S. & Lin, H.X. (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nature Genetics*, **37**, 1141-1146.

Sibly, R.M. & Calow, P. (1989) A life-cycle theory of responses to stress. *Biological Journal of the Linnean Society*, **37**, 101-116.

Sibly, R.M. (1996) Effects of pollutants on individual life histories and population growth rates. *Ecotoxicology-a hierarchical treatment* (eds M.C. Newman & C.H. Jagoe), pp. 197-223. Lewis, Boca Raton, FL.

Sih, A., Bell, A.M. & Kerby, J.L. (2004) Two stressors are far deadlier than one. *Trends in Ecology and Evolution*, **19**, 274-276.

Snape, J.R., Maund, S.J., Pickford, D.B. & Hutchinson, T.H. (2004) Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. *Aquatic Toxicology*, **67**, 143-154.

Snell, T.W. (1999) Toxicity and sexual reproduction in Rotifers: reduced resting egg production and heterozygosity loss. *Genetics and ecotoxicology* (ed V.E. Forbes), pp. 169-186. Taylor & Francis, Philadelphia.

Stark, J.D., Banks, J.E. & Vargas, R. (2004) How risky is risk assessment: The role that life history strategies play in susceptibility of species to stress. *Proceedings of the National Academy of Sciences, USA*, **101**, 732-736.

Stearns, S.C. (1987) *The evolution of sex* Oxford University Press, Oxford.

Walker, C.H. (2006) Ecotoxicity testing of chemicals with particular reference to pesticides. *Pest Management Science*, **62**, 571-583.

Weider, L.J. (1993) A test of the general purpose genotype hypothesis - differential tolerance to thermal and salinity stress among *Daphnia* clones. *Evolution*, **47**, 965-969.

Weismann, A. (1889) The significance of sexual reproduction in the theory of natural selection. *Essays on heredity and kindred biological subjects* (eds E.B. Poulton, S. Schönland & A.E. Shipley), pp. 254-338. Oxford University Press, Oxford.

West, S.A., Lively, C.M. & Read, A.F. (1999) A pluralist approach to sex and recombination. *Journal of Evolutionary Biology*, **12**, 1003-1012.

Williams, G.C. (1975) *Sex and evolution* Princeton University, Princeton, New Jersey.

Yue, B., Xue, W.Y., Xiong, L.Z., Yu, X.Q., Luo, L.J., Cui, K.H., Jin, D.M., Xing, Y.Z. & Zhang, Q.F. (2006) Genetic basis of drought resistance at reproductive stage in rice: Separation of drought tolerance from drought avoidance. *Genetics*, **172**, 1213-1228.

Summary

Human activities increasingly affect the natural environment. Consequently, many organisms are confronted with environmental conditions different from the ones they evolved in and experience stress. Environmental stress affects various levels of biological organization (e.g. cell, life-histories, population, community, ecosystem) and concerns various time-spans. Therefore, to understand the mechanisms behind stress responses and the consequences for natural systems requires incorporation of multiple scientific disciplines.

In this thesis I incorporate the frameworks of ecology and evolutionary genetics to investigate the effects of long-term stress on natural populations. In addition, I provide the resources to study genetic basis of stress-related traits within QTL approach. Nematodes were selected as the research subjects because of their wide distribution, importance for major ecological processes, such as decomposition, and the ease of laboratory handlings and genetic analysis.

The major part of the presented research focused on the long-term effects of copper and pH stress on natural populations of the parthenogenetic nematode *Acrobeloides nanus*. Chapters 2, 3 and 4 present an extensive analysis of the populations from an experimental field where copper and pH treatments were applied in a factorial design approximately 20 years ago. At first, I considered effects of the combined stressors on population biomass and functional parameters such as secondary production and biomass turnover rate. Despite the fact that multiple anthropogenic stressors are common in human-dominated environment, the knowledge of their possible synergistic effects on population-level parameters is very limited. The investigation reported in chapter 2 is possibly the first study using a controlled experimental system in the field to disentangle the interactive effects of stressors on population parameters. The results indicated negative synergistic effects of high copper level and low pH on population secondary production and biomass turnover in *A. nanus*. Surprisingly, the biomass of *A. nanus* showed no negative effects under the same conditions. Overall, these findings demonstrated the impact of interactive stress effects and suggested that functional population parameters might be more sensitive to combination of stressors than the measures such as biomass or population abundance. Consequently, I concluded that realistic risk assessment would benefit from the analyses of combined stressors effects and incorporation of functional population measures. In chapter 3, I focused on the question whether the observed changes in life-history traits in *A. nanus* were the result of adaptation to local copper and pH conditions. Reciprocal transplant- and reaction norm experiments indicated that the populations from two extreme stress treatments underwent adaptive divergence within approximately 20 years of exposure. I interpreted these results from the perspective of evolutionary biology of asexual species and concluded that they contradict the general view of low adaptive potential of asexual eukaryotes. They also

demonstrate that the population structure and distribution of asexual species might be shaped by local adaptation events. In chapter 4, I tested whether the adaptive changes in life-history traits of *A. nanus* were accompanied by underlying changes in genetic architecture represented by genetic variance-covariance matrix (**G**). Within evolutionary genetics, the concept of **G** matrix is central as it provides the framework for the analysis of phenotypic evolution under the assumption of **G** stability over time. It is acknowledged that genetic drift or strong selection such as stress might lead to evolution of **G** matrices, however the time-spans and conditions promoting these changes in natural populations remain unknown. The comparisons of **G** matrices for life-history traits of *A. nanus* indicated a profound divergence of **G** structure as a response to divergent selection imposed by field treatments and pointed to a less distinct divergence of **G** matrices within the treatments that are likely to be attributed to drift. Because all the detected changes took place within 20 years of the existence of the experimental field, I concluded that genetic architecture might evolve rapidly in natural populations. These results suggest also that strong stress might enhance this process and that the observed high dynamics of **G** structure is likely to represent a general feature of asexual species.

The second part of the presented research concerned the genetic bases of stress-related and other complex traits in *Caenorhabditis elegans*. Although there is a growing interest for incorporating QTL methods to the mainstream genetic approaches in this model species, the lack of powerful resources hampers the advancements within this field. In chapter 5, I reported the construction of a permanent, genome-wide library of near-isogenic lines (NILs) from two parental lines, the Hawaiian line CB4856 and the Bristol line N2. All of the 87 NILs have a single, short and homozygous segment of the CB4856 genome introgressed into N2 background and in total the introgressed segments cover at least 95% of the genome length. In the same chapter, I presented the analysis of one of the stress-related traits in *C. elegans*, clumping behaviour, using both recombinant inbred lines (RILs) and selected NILs which resulted in detection of a novel locus responsible for natural phenotypic variation in this trait. Overall, I concluded that the properties of this library allow for more efficient and accurate QTL localization and facilitate gene identification.

In chapter 6, I discussed the most relevant findings reported in this thesis and considered them in the context of fundamental problems (evolution of sexual reproduction) and applied issues (the use of clonal organisms for toxicity testing). I devoted also one section of this chapter to discuss the role of QTL mapping in stress-related research. Finally, I made some suggestions regarding future research directions and risk assessment strategies.

Samenvatting

De mens beïnvloedt in toenemende mate zijn natuurlijke omgeving. Als gevolg hiervan worden vele organismen geconfronteerd met milieuomstandigheden die afwijken ten opzichte van het milieu waarin ze geëvolueerd zijn en ondervinden hierdoor stress. Milieustress heeft effect op verschillende niveaus van de biologische organisatie (zoals cel-, individu-, populatie-, levensgemeenschap- en ecosysteemniveau) en heeft betrekking op verschillende tijdsspannes. Inzicht in de onderliggende mechanismen van de stress response en de gevolgen voor natuurlijke systemen vereist een bijdrage van diverse onderzoeksdisciplines.

In dit proefschrift bestudeer ik de effecten van lange-termijn stress op natuurlijke populaties vanuit een ecologisch en evolutionair genetisch perspectief. Parallel hieraan presenteer ik een nieuwe bron voor genetische onderzoek aan de hand waarvan de genetische basis van complexe eigenschappen (zoals stress response) kan worden geanalyseerd met behulp van QTL-methoden. Voor het onderzoek zijn nematoden gebruikt omdat ze bijna overal voorkomen, een belangrijke rol spelen in ecologische processen zoals decompositie en omdat ze makkelijk in het laboratorium te bestuderen zijn.

Een belangrijk deel van het onderzoek richt zich op de lange-termijn effecten van koper en pH op natuurlijke populaties van de parthenogenetische nematode *Acrobeloides nanus*. In hoofdstukken 2, 3 en 4 wordt een diepgaande analyse gegeven van de effecten op populaties in een experimenteel veld waar 20 jaar geleden koper en pH in een factoriele design zijn aangebracht. Eerst bestudeerde ik de gezamenlijke effecten op de biomassa van de populatie en functionele parameters zoals secundaire productie en biomass turnover rate.

Hoewel er in milieus die door mensen worden gedomineerd veelal verschillende antropogene stressoren aanwezig zijn, is er weinig kennis omtrent de mogelijke synergistische effecten op populatieniveau. Hoofdstuk 2 beschrijft de eerste studie waarbij gebruik wordt gemaakt van een experimentele veldopzet voor het onderzoek naar de gecombineerde effecten van verschillende stressoren op populatie parameters. Bij relatief hoge koperconcentraties en lage pH-waarden werden er synergistische effecten waargenomen op secundaire productie en biomassa turnover in *A. nanus*. Verrassend genoeg werden bij deze condities geen negatieve effecten gevonden op de biomassa. Deze resultaten suggereren dat functionele populatie parameters gevoeliger zijn voor koper en pH-waarde dan de biomassa of grootte van de populatie. Op basis van deze resultaten kan geconcludeerd worden dat de ecologische risicoanalyse van milieuvreemde stoffen gebaat is bij de analyse van gecombineerde effecten en de incorporatie van functionele populatie parameters.

In hoofdstuk 3 wordt bestudeerd in hoeverre de waargenomen effecten op life-history kenmerken in *A. nanus* het gevolg zijn van adaptatie aan koper en pH-waarde. De resultaten van reciproke transplantatie- en reactienormexperimenten laten zien dat

de populaties afkomstig van twee extreme stressbehandelingen adaptatie laten zien aan de stressvolle omstandigheden na 20 jaar blootstelling. Deze uitkomsten worden bediscussieerd in de context van de evolutie van asexuele soorten. Geconcludeerd kan worden dat, in tegenstelling tot wat voor kort werd aangenomen, asexuele eukaryoten zich snel via adaptatie kunnen aanpassen. De resultaten laten ook zien dat de populatiestructuur en verspreiding van asexuele soorten bepaald worden door lokale adaptatie. In hoofdstuk 4 onderzoek ik of de adaptieve aanpassingen van life-history kenmerken in overeenkomst zijn met de genetische structuur zoals beschreven wordt door de variatie-covariatie matrix **G**. In de evolutionaire genetica wordt de matrix **G** gebruikt voor de analyse van fenotypische evolutie onder de aanname dat **G** stabiel is in de tijd. Algemeen wordt aangenomen dat **G** verandert onder invloed van genetische drift of selectie maar de tijdspanne waarin en de condities waaronder dit gebeurt zijn niet bekend. Vergelijking van **G** voor verschillende life-history eigenschappen laten een duidelijke divergentie zien die veroorzaakt wordt door selectie en niet door drift. Dit is een snelle genetische aanpassing, aangezien deze veranderingen plaatsvonden gedurende een relatief korte periode van 20 jaar. Dit suggereert dat zware stress dit proces versterkt en dat de waargenomen dynamiek in de structuur van **G** een algemene karakteristiek is van asexuele soorten.

Het tweede deel van mijn proefschrift richt zich op de genetische basis van stress gerelateerde-, en andere complexe eigenschappen in de nematode *Caenorhabditis elegans*. Alhoewel er een toenemende interesse is in de toepassing van QTL-methodieken voor deze modelsoort is er momenteel weinig gereedschap beschikbaar om dit onderzoek uit te voeren. In hoofdstuk 5 beschrijf ik de ontwikkeling van een permanente, genoom-brede bibliotheek van bijna isogene lijnen (NILs) afkomstig van een kruising tussen CB4856 uit Hawaii en N2 uit Bristol, UK. Alle 87 NILs hebben een kort homozygoot segment van het CB4856 genoom in een N2 achtergrond. Het totale CB4856 segment omvat minstens 95% van het totale CB4856 genoom. In dit hoofdstuk analyseer ik een stress-gerelateerde eigenschap van *C. elegans*, namelijk groepsgedrag. Hierbij maakte ik gebruik van zowel NILs als recombinant inteelt lijnen (RILs); dit resulteerde in de identificatie van een nieuw locus dat geassocieerd is met natuurlijke variatie in deze eigenschap. De conclusie is dat deze NILs een belangrijke bron kunnen zijn voor de detectie en identificatie van causaal gerelateerde genen.

In hoofdstuk 6 bediscussieer ik de meest relevante bevindingen van dit proefschrift en belicht ze vanuit verschillende invalshoeken, waaronder de evolutie van sexuele reproductie en het gebruik van klonale organismen voor toxiciteitstudies. Ook wordt aandacht gegeven aan het gebruik van QTL-benaderingen voor onderzoek naar stress-gerelateerde eigenschappen. Tenslotte doe ik een aantal suggesties voor toekomstig onderzoek en voor methoden die gebruikt kunnen worden in risicoanalyse.

Acknowledgements

Writing acknowledgements is probably one of the most awaited and satisfying moments in the whole writing process. It marks the end of the long way from freshly born ideas to fully shaped text and it introduces us as humans and emphasizes personal aspects of our work. It is, thus, time to get less formal and humanize this thesis…

First of all, I would like to thank Jan Kammenga and Jaap Bakker. At the very beginning of my 'research journey' you showed your trust in me and introduced me to the Nematology team. Jan, I'm really happy that you were my supervisor and co-promotor. If the most important characteristic of superb supervisors is that they facilitate the research and the development of young researchers, you are definitely one of the examples falling within the category. Now I know that it is quite difficult to write the thesis which embraces the topics of very different disciplines such as ecology, evolutionary biology and quantitative genetics but, I guess, it is even more difficult to provide supervision covering these areas. Thank you for your open attitude and flexibility.

I would like to direct my special thanks to Ryszard Laskowski and Paulina Kramarz. Since the time we met I've been inspired by your passion for research. Your direct and indirect influence on my professional and private life is enormous.

Long time ago somebody wrote: 'The true way to render ourselves happy is to love our work and find in it our pleasure'. Nowadays, I could add to it: 'quality of work relies upon quality of coffee breaks'. How we feel is related very strongly to how we work (and *vice versa*) and the role of colleagues in this process shouldn't be underestimated. I'm very grateful to all my colleagues for all coffee breaks, lunches, birthday cakes, bbqs, Christmas lunches and other social activities which added extra quality to the routine of working days. Thank you also for fantastic atmosphere during 'normal' working hours. Marie-José, Martijs and Evert, you shared with me your experiences and I could always count on your help. It was never boring with you around. Olga, I'm glad that we accompanied each other on the way to the doctor degree. Joost, the cooperation with you is superb and I appreciate a lot all the work you've done. Sven, Rikus and Lisette, I could always count on you when it came to organization and logistic problems, thanks!!! Kasia, my new roommate, I wouldn't expect that it can be actually nice to share the office with somebody else than my husband! Nematology is also the place where I met some of my dear friends. Aneta, Ula, Patrick, Pikutek, Kamila, Pisiek, Tomek, Ania, we shared a lot of great time together, I'm really grateful for that! It would not be fair if I did not mention Piotrek, Ania M., Dominika and Sewer who I met out of Nematology. Thanks for all social activities together! Richard, thanks for your useful comments on the thesis! During my PhD training I was also lucky to work with several fantastic students. Marcin, Elske, David, Irena, Rebeca, Bas, Emilie, Piotr and Ana, I was impressed by your dedication

and enthusiasm for research. Good luck with your future plans! I shouldn't forget, of course, about my paranymphs, Marysia and Aneta. Marysia, I've always admired your pragmatic attitude and incredible organization skills. Anetka, you are a great companion and I always enjoy your charming personality.

Leaving our country means leaving people who are important to us. Luckily, with modern communication possibilities and transport facilities, distances have become smaller. My dear friends, Magda, Agusia, Monika, Wojtek, Asia, Rysiek, Paulina, Maciek, Radek, Ziggy, Lila, Ola, Pawel… thanks to you whenever I come to Kraków I feel at home.

Kochana rodzinko, Mamo, Tato i Tomku, dziękuję, że pozwoliliście mi realizować własne marzenia. Bez Waszego wsparcia napisanie tej pracy nigdy nie byłoby możliwe.

Ik wil ook mijn schoonfamilie speciaal bedanken voor hun steun en interesse in mijn werk en voor de gezellige tijd samen. Jullie geven mij het gevoel dat ik erbij hoor en daarvoor ben ik buitengewoon dankbaar.

My first day in Wageningen as a PhD student was special not only because I started the work on this thesis but also because I met my husband. André, in a miraculous way you combine the best qualities of a critical scientific advisor, the best friend and a fantastic partner in life. You deserve the greatest THANKS in the world!

Curriculum Vitae

Agnieszka Doroszuk was born on the 1st of October 1976 in Bielsko-Biała, Poland. In 1995, she completed her high school studies and moved to Kraków to study biology at the Jagiellonian University. In the years 1996-2000, she was awarded with Jagiellonian University scholarship for excellent students. The academic year of 1997-1998 she spent out of academia traveling in India and Nepal and working at archaeological excavations near Kraków as a research assistant. Afterwards she continued her studies. In 1999, she spent six months in the Netherlands working on a project at the Laboratory of Nematology at Wageningen University. She was awarded twice (1999 and 2000) with Minister of Education Scholarship for best Polish students. In the year 2001, Batory Foundation awarded her with a three-months scholarship at Oxford University, where she worked under the guidance of dr. Paul Reiney and dr. Angus Buckling. She returned to Kraków in April 2001 and completed her university education in July with a MSc degree in Biology (with honors). Shortly after graduation, she received Fiat Polska award for excellent students. In the year 2002, she was appointed as a PhD student at the Laboratory of Nematology at Wageningen University.

List of publications

Articles

Doroszuk, A., te Brake, E., Crespo-Gonzalez, D. and Kammenga, J.E. (2007) Response of secondary production and its components to combinations of environmental stressors in nematode field populations. *Journal of Applied Ecology* **44(2):** 446

Doroszuk, A., Wojewodzic, M.W. and Kammenga, J.E. (2006) Rapid adaptive divergence of life-history traits in response to abiotic stress within a natural population of a parthenogenetic nematode *Proceedings of the Royal Society. B: Biological Sciences,* **273(1601):** 2611-2618.

Kammenga, J.E., Doroszuk, A., Riksen, J.A.G., Hazendonk, E., Spiridon, L, Petrescu, A., Tijsterman, A., Plasterk, R.A.H. and Bakker, J. (2007) A *C. elegans* wild-type defies the temperature-size rule due to a single nucleotide polymorphism in tra-3. *PloS genetics, in press*

Gutteling, E.W., Doroszuk, A., Riksen, J.A.G., Prokop, Z., Reszka, A. and Kammenga, J.E. (2007) Environmental influence on the genetic correlations between life-history traits in *Caenorhabditis elegans. Heredity, in press*

Tobor-Kaplon, M.A., Holtkamp, R., Scharler, U.M., Doroszuk, A., Kuenen, F.J.A., Bloem, J. and de Ruiter, P.C. (2007) Can the information idices (Ecosystem Network Analysis) be used as indicators of environmental stress in terrestrial ecosystems*? Ecological modelling, accepted*

Van der Wurff, A.W.G., Kools, S.A.E.,. Boivin, M.E.Y, van den Brink, P.J., van Megen, H.H.M,. Riksen, J.A.G, Doroszuk, A. and Kammenga, J.E. (2007) Type of disturbance and ecological history determine structural stability. *Ecological Applications* **17**:190–202.

Book contributions, reports

Kammenga, J.; Arts, M.J. and Doroszuk, A. (2000): Multi-generation effects at the population level: Fitness maximisation and optimal allocation in a nematode In: *Demography in Ecotoxicology* eds. Jan Kammenga and Ryszard Laskowski. Wiley & Sons, Chichester

Van der Wurff, A.W.G., Kools, S.A.E.,. Boivin, M.E.Y, van den Brink, P.J., van Megen, H.H.M,. Riksen, J.A.G, Doroszuk, A. and Kammenga, J.E. (2007) Metals affect secondary stress sensitivity of nematode field populatins, *RIVM rapport 860706002*

Conference symposia proceedings and meetings

Doroszuk, A., Wojewodzic, M., te Brake, E. and Kammenga, J.E. (2004) Adaptive divergence of nematode field populations as a response to long term stress. Fribourg, Switzerland, *Experimental Evolution Workshop* (2004, October 4).

Doroszuk, A., Wojewodzic, M., te Brake, E. and Kammenga, J.E. (2004) Adaptive divergence of nematode populations as a response to long term stress. In *Netherlands Scientific Symposium Soil & Water, Zeist, 2-3 June 2004*. Zeist.

Doroszuk, A., Wojewodzic, M.W. and Kammenga, J.E. (2005) Rapid evolution of G matrices in nematode field populations. In *10th Congress of European Society for Evolutionary Biology, Krakow, Poland, 15-20 August 2005* Krakow, Poland

Doroszuk, A., Fradin, E., Van de Waterbeemd, B.,Riksen, J.A.G. and Kammenga, J.E. (2005) CB4856/N2 Introgression Lines of *Caenorhabditis elegans*- a permanent population for QTL fine-mapping. *European worm meeting*, Hersonissos, Crete

Doroszuk, A., Fradin, E., Van de Waterbeemd, B.,Riksen, J.A.G. and Kammenga, J.E. (2005) CB4856/N2 Introgression Lines of *Caenorhabditis elegan*s- a permanent population for QTL fine-mapping. *4th Annual Meeting of the Complex Trait Consortium*: Groningen (June 26 -29, 2005).

Doroszuk, A., Fradin, E., Waterbeemd, B., Riksen, J.A.G. and Kammenga, J.E. (2005) CB4856/N2 Introgression Lines of *Caenorhabditis elegans*- a permanent population for QTL fine-mapping. *Evolutionary biology of C. elegans workshop*, Oeiras, Portugal

Doroszuk, A., Lukasik, P., and Kammenga, J.E. (2005) Genetic architecture of fitness in the nematode *Caenorhabditis elegans*. *10th Congress of European Society for Evolutionary Biology*: Krakow, Poland (August 15 – 20, 2005).

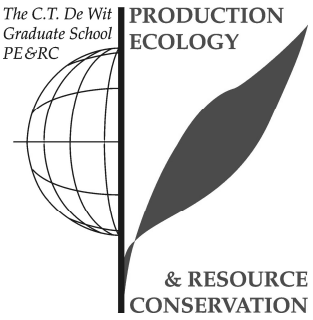
Doroszuk, A., Wojewodzic, M.W. and Kammenga, J.E. (2005) Rapid evolution of G matrices in nematode field populations under stress. *Netherlands Scientific Symposium Soil & Water:* Zeist (June 1 – 2, 2005).

Doroszuk, A., Wojewodzic, M. and Kammenga, J.E. (2003) Effects of long-term exposure to copper on the performance of nematode populations. In *Proceedings 2nd national scientific soil symposium BodemDiep* Gouda: Stichting kwaliteitsbevordering bedrijfsgezondheidszorg.

Holterman, M.H.M.,Doroszuk, A.,Helder, J. and van der Wurff, A.W.G. (2006) A comparative analysis of traits involved in stress tolerance in nematodes. In *European Society of Nematologists XXVIII International Symposium, Blagoevgrad, Bulgaria, 5-9 June 2006* . Blagoevgrad, Bulgaria.

Tobor-Kaplon, M.A., Holtkamp, R., Scharler, U.M.,Doroszuk, A., Kuenen, F.J.A.,Bloem, J. and de Ruiter, P.C. (2006) Quantifying the impact of stress on soil ecosystem development using Information Indices (Ecosystem Network Analysis). In *Controversies and solutions in environmental sciences; abstract book SETAC Europe 16th annual meeting* (pp. 91). Brussels (Belgium): SETAC.

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 (5.6 credits)

- Effects of abiotic stress on the structure, stability and functioning of populations of soil organisms (2002)

Post-Graduate Courses (7 credits)

- Special topics in ecotoxicology: community ecotoxicology; SENSE (2002)
- Soil Ecology : linking theory to practice; FE, SENSE, PE&RC (2003)
- Basic and advanced statistics; PE&RC (2003/2004)

Deficiency, Refresh, Brush-up and General courses (3,3 credits)

- QTL course (2002)
- Isotope course; Larenstein (2002)
- Introductory course in nematology: molecular identification; Nematology (2005)
- PhD competence assessment; PE&RC (2004)

PhD Discussion Groups (8.4 credits)

- Genetic resources and diversity in production ecology-discussion group (2002-2003)
- National C. elegans meeting; Rotterdam (2002)
- Ecogenomics- one day symposium; Amsterdam (2003)
- Current themes in ecology: experimental evolution, fundamental and applied (2004)
- Current themes in ecology: invasive species (2004)
- Bodem Diep, National Scientific Soil Symposium (2002/ talk, 2003)
- Soil and Water, Netherlands Scientific Symposium (talk, 2004/ 2005)

PE&RC Annual Meetings, Seminars and Introduction Days (3.6 credits)

- PE&RC annual meeting: Ethics in science (2002); Global climate change and biodiversity (2003); Biological disasters (2004); The truth of science (2005)
- PE&RC symposium: Ecological and Evolutionary Genomics (2005)
- Cutting-Edge Ecology (2005)
- PE&RC weekend (2002/ 2003)

International Symposia, Workshops and Conferences (11.5 credits)

- Population growth rate: determining factors and role in population regulation; Royal Society, London, UK (2002)
- Ninth congress of European Society of Evolutionary Biology; Leeds, UK (2003)
- Experimental evolution workshop; Fribourg, Switzerland (talk, 2004)
- Complex trait consortium; Groningen (2005)
- Ecogenomics; Kansas (2005)
- Tenth congress of European Society of Evolutionary Biology; Krakow, Poland (talk, 2005)
- European worm meeting; Hersonisos, Crete, Greece (talk, 2006)
- Caenorhabditis elegans evolutionary workshop; Portugal (2006)

Laboratory Training and Working Visits (2 credits)

- Tiling arrays technology in Caenorhabditis elegans; Sanger Institute, Hinxton, Cambridge (2006)
- New strategies to investigate genetic bases of behaviour in Caenorhabditis elegans; The Medical Research Coucil, Cambridge (2006)

Printed by: Gildeprint B.V., Enschede

Cover: Agnieszka Doroszuk

The research described in this thesis was carried out at the Laboratory of Nematology, Wageningen University, Binnenhaven 5, 6709 PD Wageningen, the Netherlands.

Financial support was provided by the TRIAS Programme (TRIpartite Approach to Soil system processes) of Delft Cluster, SKB (Foundation for development and transmission of soil knowledge) and NWO (the Netherlands Organisation for Scientific Research), as project 835.80.008.