

Micro-evolution of toxicant tolerance: from single genes to the genome's tangled bank

Nico M. van Straalen · Thierry K. S. Janssens · Dick Roelofs

Accepted: 1 March 2011 / Published online: 18 March 2011
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Abstract Two case-studies published 55 years ago became textbook examples of evolution in action: DDT resistance in houseflies (Busvine) and the rise of melanic forms of the peppered moth (Kettlewell). Now, many years later, molecular studies have elucidated in detail the mechanisms conferring resistance. In this paper we focus on the case of metal tolerance in a soil-living arthropod, *Orchesella cincta*, and provide new evidence on the transcriptional regulation of a gene involved in stress tolerance, metallothionein. Evolution of resistance is often ascribed to *cis*-regulatory change of such stress-combating genes. For example, DDT resistance in the housefly is due to insertion of a mobile element into the promoter of *Cyp6g1*, and overexpression of this gene allows rapid metabolism of DDT. The discovery of these mechanisms has promoted the idea that resistance to environmental toxicants can be brought about by relatively simple genetic changes, involving up-regulation, duplication or structural alteration of a single-gene. Similarly, the work on *O. cincta* shows that populations from metal-polluted mining sites have a higher constitutive expression of the cadmium-induced metallothionein (*Mt*) gene. Moreover, its promoter appears to include a large degree of polymorphism; *Mt* promoter alleles conferring high expression in cell-based bioreporter assays were shown to occur at higher frequency in populations living at polluted sites. The case is consistent with classical examples of micro-evolution through altered *cis*-regulation of a key gene. However, new data on qPCR analysis of gene expression in homozygous genotypes with both reference and metal-tolerant genetic backgrounds,

show that *Mt* expression of the same *pMt* homozygotes depends on the origin of the population. This suggests that *trans*-acting factors are also important in the regulation of *Mt* expression and its evolution. So the idea that metal tolerance in *Orchesella* can be viewed as a single-gene adaptation must be abandoned. These data, added to a genome-wide gene expression profiling study reported earlier shows that evolution of tolerance takes place in a complicated molecular network, not unlike an internal tangled bank.

Keywords Evolution · Tolerance · Stress · Heavy metals · Metallothionein · Genetics · Soil invertebrates · *Orchesella cincta*

Introduction

More than five decades ago the first studies were published demonstrating that anthropogenic pollution can act as a selective force causing micro-evolution in wild populations. Busvine (1954) showed that houseflies could develop a genetically determined resistance against organochlorine pesticides. A remarkable result of this report was that the houseflies were resistant not only to DDT, but to a whole group of organochlorine compounds. At about the same time, Kettlewell (1955) first demonstrated that the melanic form of the peppered moth was increasing in frequency relative to the black-and-white speckled wild type, due to a camouflage advantage on darkened tree barks covered with soot from air pollution.

These two examples are widely accepted as the first case-studies illustrating the principle of “evolution through pollution”. They have entered textbooks of evolution as concrete evidence supporting the action of natural selection

N. M. van Straalen (✉) · T. K. S. Janssens · D. Roelofs
Department of Ecological Science, VU University, De Boelelaan
1085, 1081 HV Amsterdam, The Netherland
e-mail: nico.van.straalen@falw.vu.nl

in the wild. Although, the term ecotoxicology had not yet been coined in the 1950s, on hindsight the studies of Busvine and Kettlewell can be considered truly ecotoxicological. They show that ecotoxicology can contribute importantly to fundamental biological disciplines such as evolutionary biology.

The reason why these studies were so instrumental as showcases of micro-evolution is that environmental toxicants often represent strong stress-inducing factors in organisms exposed to them. Stress in general may be defined as an internal condition, deviating from normal, of an organism that finds itself out of its ecological niche (Van Straalen 2003). Organisms can usually survive such conditions temporarily by staging a variety of protective mechanisms, e.g. the production of heat shock proteins, upregulated oxidative stress defence and activation of the immune system (Kültz 2005). The study of these mechanisms is the domain of environmental stress physiology.

One way to cope with persistent or recurring stress is to evolve tolerance, that is, to enhance the physiological capabilities in such a way that what was stress before is not experienced as stress anymore. This will occur if there is genetic variation between individuals in the stress-combatting mechanisms. Individuals with gene variants that allow them to better neutralize the stress, prevent adverse effects or quickly repair the damage, will leave more offspring in the next generation and will increase in frequency, and so will these gene variants.

This paper discusses the mechanisms that may underlie the evolution of tolerance to environmental toxicants. A case study on metal tolerance in a soil-living invertebrate is presented to test the hypothesis that tolerance is due to the regulation of a single-gene. Inspired by the results we suggest that, on the molecular level, evolution is taking place in a complex network of gene interactions involving genome-wide changes in gene expression.

Single-gene based tolerance

One of the mechanisms receiving quite some attention by which toxicant tolerance can be achieved, is increased expression of stress-combatting genes (Fig. 1). Often this involves changes in the promoter of the gene, causing altered *cis*-regulation. Nucleotide substitutions, insertion and deletions in the promoter sequence may effect transcription in many different ways. For example, changes in the arrangement of transcription factor-binding sites may increase or decrease the binding affinity of toxicant-induced transcriptional regulators and thus alter the expression of downstream genes.

A well-investigated example illustrating this type of mechanism is described by Daborn et al. (2002). These

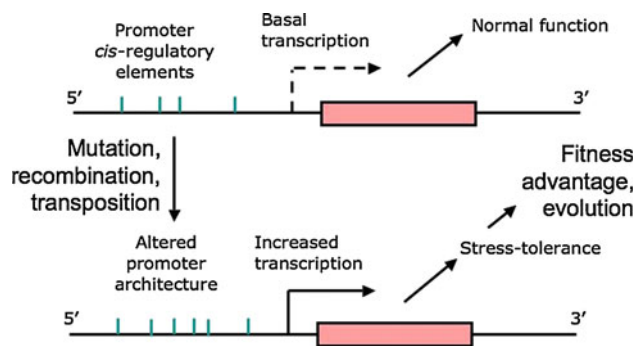


Fig. 1 Cartoon of single-gene-based tolerance evolution. Tolerance may be achieved by mutations in the architecture of the promoter of a gene encoding a protein contributing to stress tolerance. Such *cis*-regulatory change may be due to alterations in the number or relative position of transcription factor-binding sites caused by single nucleotide substitutions, indels, recombination or transposition. If these mutations result in constitutive or induced over-expression of stress-combatting genes they may cause a fitness advantage to the phenotype and be favored by selection

authors were able to show that resistance to DDT in fruitflies could be linked to over-expression of a single-gene, *Cyp6g1*, which encodes a cytochrome P450 enzyme that will degrade DDT to a non-toxic metabolite. In wild type flies *Cyp6g1* has a low rate of expression, however, in the DDT-resistant line a mobile genetic element of the Accord type is inserted in the promoter of the gene. This brings *Cyp6g1* under very active transcriptional control and the gene is over-expressed several times. The fact that this tolerance was proven to be due to a single promoter allele is a unique aspect of the study. Later work has shown that several other mutations have occurred at the *Cyp6g1* locus. In an “adaptive walk” of at least four steps, including duplication of the gene plus its promoter, and insertion of another mobile element, each new allele provided a slightly better DDT resistance. So the locus was changed profoundly, not in one sweep but in several small steps (Schmidt et al. 2010).

Over-expression of a resistance gene by *cis*-regulatory change is a relatively simple mechanism that is expected to come without appreciable metabolic costs or negative pleiotropic effects. The question may therefore be raised, is this type of tolerance a common phenomenon for evolution in the wild? This question relates to an issue of wider importance in evolutionary biology. Until recently, there was a general agreement among evolutionary biologists that changes in the body plan of animals are mostly due to altered *cis*-regulation of developmentally important genes, e.g. *Hox* genes (Carroll et al. 2005). The coding regions of such genes, being very conservative across a large part of the tree of life, would not be sufficiently flexible to explain the large changes in animal body plans, hence the “primacy of regulatory evolution” (Carroll et al. 2005,

Chapter 8). On the other hand, the adaptive responses studied by physiologists and ecologists (e.g. low temperature adaptation caused by amino acid replacements in lactate dehydrogenase, Field and Houseman 2004) would be mostly due to substitutions in coding regions of genes, i.e. structural evolution.

These general statements, emphasizing the importance of *cis*-regulatory change in the evolution of body plans and structural evolution in stress adaptation, have been questioned by Hoekstra and Coyne (2007). After a review of the literature these authors concluded that the overall enthusiasm for *cis*-regulatory change as the prime mechanism of evolution is not borne out by the evidence. In fact there are many examples of *cis*-regulatory change in physiological adaptation and structural changes in body plan evolution. We will see in the present paper that indeed the situation is much more complex than thought before.

Heavy metal tolerance in a soil-living hexapod

Janssens et al. (2008) investigated a presumed single-gene based tolerance in the soil-dwelling invertebrate, *Orchesella cincta*. This hexapod, belonging to the class of Collembola (springtails), is very common in woodlands and forests across North-West Europe. The density, averaged across the seasons, was estimated as 1,500 individuals per m² in a young woodland, although there may be many more at times of population peaks (Van Straalen 1989). The widespread occurrence in a great variety of habitats, makes *O. cincta* an interesting ecological model. The species, being of separate sex, easily breeds in the laboratory, lends itself to controlled crosses and is therefore also suitable for studies in evolutionary genetics.

Orchesella cincta is found in many heavily polluted woodland soils where other species of invertebrate are absent. At industrial and mining sites the concentrations in soil and litter are often far above the no-effect levels established in toxicity tests with laboratory cultures. The question was raised how the field-living animals can survive toxicant exposures that inhibit growth and reproduction of laboratory cultures and populations living in clean environments.

One factor contributing to metal tolerance is over-expression of a metal-induced and metal-binding protein, metallothionein. We have demonstrated that *O. cincta* metallothionein (*Mt*) is a single-copy gene which has a highly upregulated expression when exposed to cadmium in the food. In addition, the constitutive expression (shown by animals cultured in the laboratory and exposed to clean food) is correlated with the cadmium concentrations in soil and litter at the site from which the animals were sampled. Animals sampled from heavily polluted sites in Europe

such as Plombières (Belgium, a former mining site), Noyelles (France, a site polluted by metals smelting works) and Arnoldstein (Austria, also polluted by metal smelting works) have a higher constitutive *Mt* expression than reference sites at other European locations (Timmermans et al. 2005).

Subsequent work has shown that the variability of *Mt* expression can be related to polymorphisms in the proximal promoter (Janssens et al. 2007). These polymorphisms, most likely originating from extensive recombinations, were shown to have functional consequences. Nine different promoter alleles were identified that differ in the number and spacing of metal-responsive elements, the assumed binding sites for metal transcription factor (MTF). Using a luciferase-based bioreporter system in *Drosophila* S2 cell lines, Janssens et al. (2007) were able to indicate two promoter alleles (*pMtD2* and *pMtF*) that conferred a much higher expression than the “wild type”, *pMtA1*. Another allele, *pMtB*, had an expression comparable to *pMtA1*, while *pMtD1* and *pMtC* were hardly activated by cadmium. In addition, Janssens et al. (2008) performed a comparative analysis of allele frequencies in 20 different populations from sites in The Netherlands, Belgium, France and Germany, and demonstrated that the frequency of the high expresser allele *pMtD2* was correlated with the cadmium concentration in the soil, while the “wild type” allele *pMtA2* was strongly negatively correlated with soil metals.

These studies have provided convincing evidence that heavy metals in soil may act as a selective force and cause fitness differences among individuals with different metallothionein expression. The data seem to fit very well the paradigm that tolerance to environmental toxicants is brought about by increased expression of toxicant-combatting genes (Fig. 1). Here, we report on *Mt* expression in *pMt* homozygous families to test whether this is also applicable to metal tolerance in *O. cincta*.

Population-specific transcriptional regulation of metallothionein

Materials and methods

Homozygous families at the *pMt* locus were derived from laboratory cultures of *O. cincta* (Collembola, Entomobryidae) originally sampled at a reference site (Roggebotzand, the Netherlands), and a metal-polluted site (Plombières, Belgium). To genotype individual springtails, the first two segments of one antenna were amputated; at the same time, the sex of the individual was determined by inspecting the genital aperture. This procedure left the animal alive for controlled crosses. Antennal lesions are

often found in wild populations of *O. cincta* as a consequence of predation and do not seem to affect the individuals seriously (Ernsting and Fokkema 1983).

The antenna sample was crushed in chelex and stored at -20°C until further analysis. Using an RFLP-PCR protocol developed earlier (Janssens et al. 2008), all individuals were genotyped for the *pMt* locus. Homozygous males and females were paired to start a family. Selected heterozygotes were crossed and the offspring screened for homozygotes. The homozygous families obtained were cultured for at least one generation at 15°C on twigs overgrown with green algae in pots with a bottom of plaster of paris. Eventually, eight families were obtained: (1) *pMtA1* homozygotes from Roggebotzand, (2) *pMtA1* homozygotes from Plombières, (3) *pMtA2* homozygotes from Roggebotzand, (4) *pMtA2* homozygotes from Plombières, (5) *pMtC* homozygotes from Roggebotzand, (6) *pMtC* homozygotes from Plombières, (7) another family of *pMtC* homozygotes from Roggebotzand, (8) another family of *pMtC* homozygotes from Plombières.

To test the induction of metallothionein, individuals were taken from the family culture and transferred to small pots (2 cm in diameter), with a plaster bottom, while the animal was inspected daily for moulting. When a moult was noted, the animal was transferred to another small pot with a paper disk on which a paste of green algae (*Desmococcus* spp., scraped from tree bark) was placed, which was spiked with cadmium chloride (nominal Cd concentration $1\ \mu\text{mol per g}$ dry weight), or left unspiked for the controls. After 3 days of exposure, the animals were transferred to a micro-tube, snap-frozen in liquid nitrogen and stored at -80°C until molecular analysis. For every treatment three replicate samples were prepared of five pooled animals each.

The SV total RNA isolation system (Promega) was used to extract total RNA from the sample. The tissue was crushed in $250\ \mu\text{l}$ of lysis buffer, of which $50\ \mu\text{l}$ was kept for genotyping after genomic DNA extraction with the Wizard SV genomic purification system (Promega). $5\ \mu\text{l}$ of total RNA was used in the reverse transcription reaction with 200 U MMLV (Promega) and $2\ \mu\text{M}$ of oligo(dT) primer according to manufacturer's instructions. Real-time RT-PCR was performed with SYBR® Green PCR Master Mix (Applied Biosystems) and gene-specific primers (MT-163F: GGC AAA TCG CCC ACT TGTT, MT-265R: CCT TGC AGA CAC AAT CTG GACC, ARC4-67F: CCG TAA GGA TCT GTA TGC CAA CA, and ARC4-157R: GGC AGT GAT CTC CTT TTG CATC) on an Opticon 1 real-time PCR machine (MJ Research). The cycling programme was: denaturation (95°C for 10 min), two-step amplification and quantification (92°C for 15 s, 60°C for 1 min, with a single fluorescence measurement), melting curve programme ($60\text{--}90^{\circ}\text{C}$ with a heating rate of $0.1^{\circ}\text{C per s}$ and fluorescence measurements very second). The

RFU (relative fluorescence units) of cycles 3–7 were considered as background and were baseline-subtracted from the other measurements. The cycle threshold was set to 0.01 RFU. Beta-actin was chosen as an internal control for *Mt* RNA quantification (Roelofs et al. 2006), and for every replicate three pseudo-replicate measurements were done.

The data were exported to the Q-gene module in Microsoft Excel (Muller et al. 2002), which was used to calculate the mean beta-actin-normalized expression of *Mt* for every replicate. The PCR efficiency for this reaction estimated by Roelofs et al. (2006) was used. The logarithmically transformed data were analyzed by three-way analysis of variance, using SPSS version 15.0, with origin (Roggebotzand or Plombières), exposure (Cd spiked food or clean food) and genotype (homozygous *pMtA1*, *pMtA2*, or *pMtC*) as fixed factors. This was followed by a Tukey HSD post-hoc test, using the R software package, for multiple comparison of means. Tests for homogeneity of variance (*F*-tests) were executed in excel.

Results and discussion

The two replicate *pMtC* families were pooled and analyzed jointly. The logarithmically transformed expression data for the six families obtained in this way are summarized in Fig. 2. The three-way analysis of variance revealed that the most important main effects are cadmium exposure and origin. The overall effect of genotype was not significant, however, there was a significant interaction between genotype and origin (Table 1). This implies that the effect of the promoter genotype on *Mt* expression depends on the genetic background. This is seen in Fig. 2, e.g. where the animals homozygous for *pMtA2* show a strong induction of *Mt* by cadmium when derived from the reference population, but hardly any induction (but a high constitutive expression), when originating from the tolerant population.

Our data suggest that the expression of *O. cincta Mt* is regulated by two distinct influences: the *cis*-regulatory effect of the promoter as well as one or more *trans*-acting regulatory effects from still unknown genomic factors. The data are consistent with earlier research showing that the constitutive expression of *Mt*, rather than the induced expression is higher in populations living in metal-responsive elements (Timmermans et al. 2005; Roelofs et al. 2007). Interestingly, Roelofs et al. (2006) found a significant heritability (46–36%) within a reference population only for the cadmium-induced expression, not for the constitutive expression. Apparently, the *cis*-regulatory influence of the promoter contributes significantly to additive genetic variation for *Mt* expression within a population, but does not entirely explain the difference between a tolerant and a reference population.

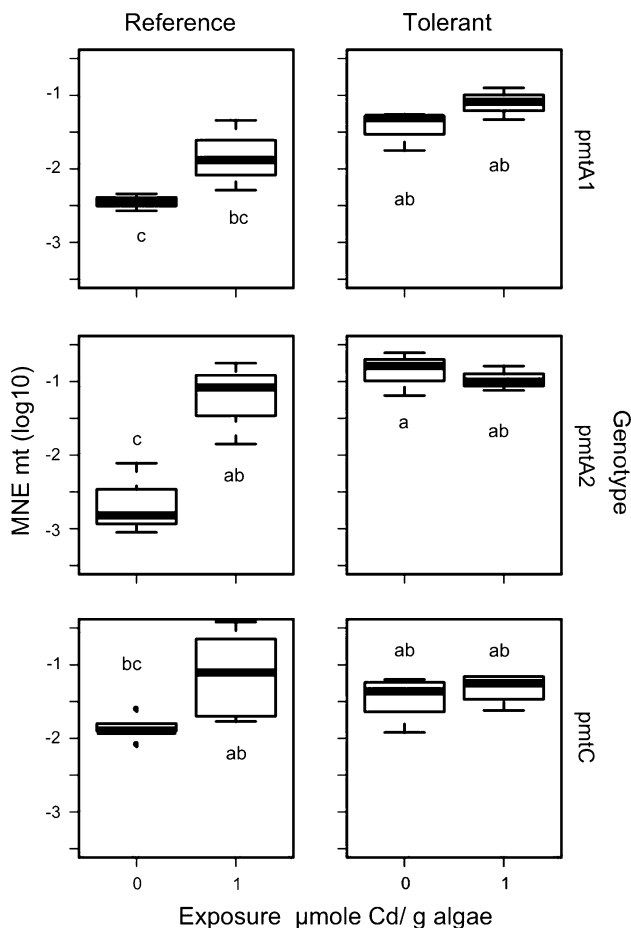


Fig. 2 Box plots representing the basal (constitutive) and induced (exposed to cadmium in green algae at a nominal concentration of 1 μmol per g dry weight) expression of metallothionein in *Orchesella cincta* homozygous for the A1, A2 and C alleles of the metallothionein promoter, in populations derived from a reference site (Roggebotzand) and a metal-polluted site (Plombières). Metallothionein gene expression was measured by qPCR, expressed relative to beta-actin and logarithmically transformed. Averages were taken of three (*pMtA1* and *pMtA2* homozygotes and six (*pMtC* homozygotes) replicates. Means within a genotype bearing the same letter (*a-c*) do not differ significantly from each other, based on the Tukey HSD post-hoc test

Some discrepancies exist between the present data and the earlier luciferase reporter assays by Janssens et al. (2007). The reduced basal and cadmium-induced expression of the *pMtCluc* construct in comparison to the *pMtAlluc* construct in the *Drosophila* S2 cell line (Janssens et al. 2007) was not observed in the present study. This could be due to regulatory differences between *D. melanogaster* and *O. cincta*. The cadmium-activated transcription factor MTF-1 from *Drosophila* might act differently on the *O. cincta* *pMt* locus than the (still putative) MTF-1 from *O. cincta* itself.

The present study was limited to three of the nine identified promoter alleles. In particular we were unable to isolate a homozygous family for the *pMtD2* allele, which is most clearly associated with metal levels in the field (Janssens et al. 2008). Further experimental work, aiming for a complete screen of *Mt* expression in all *pMt* genotypes, is necessary.

Implications for evolutionary ecotoxicology

The data presented here, and those from a recent genomics study on metal tolerance by Roelofs et al. (2009), call for a network view of pollutant-induced tolerance. Evolutionary ecotoxicologists must realize that evolution of tolerance takes place in a network of complicated genetic interactions (Fig. 3). We know from molecular biology that the regulation of a single-gene may involve more than twenty different proteins, not only the transcription initiation complex, but a host of condition-specific regulators, enhancers and repressors, all interacting with each other when binding to the regulatory elements of a gene. Each of these proteins may be subject to evolution, both in its primary structure and in the way its expression is regulated.

The network view of tolerance evolution is not unlike the tangled bank evoked by Darwin (1859) in the oft-quoted phrase on the last page of the “Origin of species”:

Table 1 Results of a three-way analysis of variance applied to log-transformed metallothionein expression (relative to beta-actin) of *Orchesella cincta*

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-statistic	P-value
Genotype	0.627	2	0.336	2.797	0.074
Exposure	2.971	1	9.971	24.713***	<0.001
Origin	4.807	1	4.807	39.993***	<0.001
Genotype \times exposure	0.110	2	0.055	0.458	0.636
Genotype \times origin	2.166	2	1.083	9.009**	0.001
Exposure \times origin	1.753	1	1.753	14.583**	0.001
Genotype \times exposure \times origin	0.663	2	0.331	2.757	0.077

Factors are: metallothionein promoter genotype (homozygous *pMtA1*, *pMtA2* or *pMtC*), exposure (cadmium exposed or exposed to clean food), and population origin (Roggebotzand or Plombières)

Significance of the *F* values is indicated by asterisks

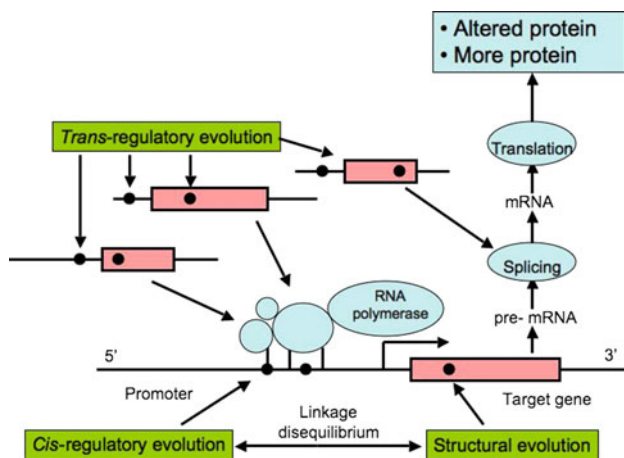


Fig. 3 Cartoon illustrating the network of molecular interactions involved with gene expression. *Black dots* indicate the multiple sites in the DNA where mutations can lead to enhanced stress tolerance. These changes can involve non-synonymous mutations in the coding region of a gene (causing an altered protein) and mutations in the 5' *cis*-regulatory region of the gene (causing more or less protein). In addition, a multitude of *trans*-acting factors can affect the expression of the gene, and mutations may affect the expression or the structure of such proteins. In addition, the phenotypic effects of these mutations are shaped by genetic interactions with other genes, e.g. due to linkage disequilibrium, pleiotropy and epistasis. So the analysis of toxicant tolerance must take a genome-wide, network perspective

“It is interesting to contemplate an entangled bank, clothed with many plants of many kinds...”. With this image, Darwin emphasized the environment as the template where evolution is operating. Dover (1999) supplemented the idea of Darwin and made a distinction between the ‘external tangled bank’ (the ecology) and the ‘internal tangled bank’ (the genome), attributing to them complementary functions in the evolutionary process. Likewise, evolutionary toxicology must abandon the idea of single-gene based tolerances and face the evolution of a genome’s tangled bank.

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