SUMMARY

Organisms can adjust the physiological status of their body by fine-tuning the expression of certain sets of genes to conditions and resources in the environment and certain stages in their life-cycle or development. This happens for a great deal at the mRNA (messenger RNA) level.

This process is called transcription and the regulation of this takes place at an upstream, non-coding region of a gene: the promoter.

By anthropogenic factors, such as environmental pollution, the genetic composition of populations can change, which causes differences in the expression patterns of specific genes, compared to populations from reference sites. These differences are favoured by natural selection when the tolerant genotypes have an increased representation in the offspring, given the environmental conditions.

The main goal of this thesis was to elucidate the potential mechanisms by which natural selection, caused by anthropogenic factors, interferes with the transcriptional regulation. For this purpose the case study of heavy metal tolerance in Orchesella cincta was used.

In preceding research evidence was found for heavy metal tolerance, especially with regard to cadmium and lead, in populations from historically polluted areas. This tolerance is manifested as an elevated excretion of cadmium and lead from the body, which comes along with increased basal and cadmium-induced levels of the mRNA of a cadmium-binding metallothionein. Therefore this thesis is invigorated on the analysis of the variation in the metallothionein promoter and its functional consequences. The above mentioned research goal is fragmented in several research questions which are dealt with in the respective chapters.

• Is there variation in the sequence of the metallothionein promoter?  Chapter 2
• Does this variation have functional consequences.  Chapter 2
• How does this variation in the promoter behave in field populations?  Chapter 3
Summary

- Are the functional differences comparable in the genetic background of different populations? \(\text{Chapter 4}\)
- Which potential transcription factors are recruited by the metallothionein promoter? \(\text{Chapter 5}\)
- Are there other genes in the *O. cincta* genome with a population-specific expression profile? \(\text{Chapter 6}\)

In Chapter 2 of this thesis the most common polymorphisms in the metallothionein promoter (pmt) of populations from Western Europe were described. Nine different alleles, forms in which a gene can appear, were discovered in the approximately 1600 bp promoter. These alleles were bearing several recombination points and contained several putative transcription factor binding sites. Transcription factors are signalling proteins which regulate the transcription of a gene at the level of the promoter, where they bind short (8-12 bp) specific DNA elements. In this way the efficiency of transcription is regulated in a positive or negative way. The most characteristic elements retrieved in metallothionein promoters from a wide range of taxa are the MRE (metal responsive element) and the ARE (anti-oxidant responsive element) were present at the pmt locus. But also the HERE (20-hydroxyecdysone responsive element) was retrieved. These elements are known from the transcriptional regulation by respectively heavy metals, oxidative stress inducers and the moulting hormone. The presence of these transcription factor binding sites did suppose that the transcription of the *O. cincta* reacts on these compounds.

The mutational spectra of the pmt locus was compared between a population from an historically polluted lead and zinc mine and a reference site. From this analysis it seemed that the diversity, measured as an excess of intermediate nucleotide polymorphisms, was higher in the adapted population and that this pattern was deviating from neutral evolution. This observation indicates a signature of natural selection, in this case *balancing selection*. This form of natural selection is characterized by an elevated genetic diversity, also at the DNA sequence level, and hence the presence of transcription factor binding DNA elements. Balancing selection can operate in the case when heterozygotes have an evolutionary advantage (overdominance) or by
the heterogeneity of the environment, in which different genotypes are favoured in specific micro-habitats.

The functional differences between the respective pmt alleles was compared in a luciferase reporter assay in cell line of the fruit fly Drosophila melanogaster. For this purpose luciferase reporter constructs were made of six abundant alleles. These constructs are artificial pieces of DNA in which the promoter of interest is cloned in front of the luciferase gene of the fire fly. The protein encoded by this gene cause light production in the presence of luciferin and ATP. In this way the activity of six promoter alleles was compared after exposure of the transiently transformed cell line with cadmium and paraquat (an oxidative stress-inducing compound). Because of the connection between the metal excretion and the moulting cycle in O. cincta the effect of the moulting hormone 20-hydroxyecdysone on the activity of the metallothionein promoter was measured as well. The induction of this promoter was most outspoken by cadmium, but diverged significantly among alleles. Paraquat was able to double the activity of five out of the six alleles, compared to the basal activity. One allele, pmtC, was not inducible by paraquat and exhibited only a limited level of cadmium induction and a low basal promoter activity. The highest cadmium-induced promoter activity was observed in the pmtD2 allele (a 20-fold of the least cadmium-inducible allele pmtC). The moulting hormone had a slightly inhibitive effect on the promoter activity of the O. cincta metallothionein promoter.

On the base of the data from Chapter 2 there was a motive for natural selection at the pmt locus of O. cincta. By means of a population genetic field study a connection between pmt allele frequencies and the heavy metal content of the environment was made. Twenty-three sites in the Netherlands, Belgium, France and Germany, from wide range of heavy metal pollution, were sampled for soil, litter and the concentration of six metals was measured. Additionally, O. cincta were sampled as well and genotyped by an RFLP (restriction fragment length polymorphism) method on their metallothionein promoter. This enzymatic method cleaves the PCR product specific way and generates an allele-specific electrophoresis pattern.

When the sampled locations were grouped according to their degree of pollution, they were best discriminated statistically by the allele frequency of the pmtD2 allele. This allele frequency was also correlated with the total cadmium content of the soil.
Moreover, this promoter allele showed the highest cadmium-inducible activities in the luciferase reporter assay (Chapter 2). These observations suggest that the \textit{pmtD2} allele has a possible evolutionary advantage in areas with cadmium pollution. However, the allele frequencies in none of the sampled populations were dominated by the \textit{pmtD2} allele. Besides this, an increase in genetic diversity was observed with the lead content of the soil. This links up with the observation of balancing selection in Chapter 2 and confirms the existence of natural selection on the transcriptional regulation of the \textit{O. cincta} metallothionein by heavy metals in the field. However, these observations do not clarify concerning the importance of polymorphisms in this promoter (\textit{cis}-regulation) in relation to polymorphisms elsewhere in the \textit{O. cincta} genome, which determine for example qualitative or quantitative features of transcription factors. These effects are know as \textit{trans}-polymorphisms or epistasis. Additionally the physical structure of the DNA in the nucleus can be modified by epigenetic effects such as histone modifications or cytosine methylations. These mechanisms could also exhibit a populations-specific pattern.

Therefore, in Chapter 4, the metallothionein mRNA levels of three \textit{pmt} alleles were compared in homozygous families from both a reference and a cadmium tolerant population. From this experiment followed that the metallothionein transcription levels are determined mainly by the origin, i.e. genetic background, of the populations, and that the \textit{pmt} alleles have a different effect on the mRNA levels in reference and tolerant populations. The most important DNA polymorphisms involved in the transcriptional regulation of the \textit{O. cincta} metallothionein are not situated in the promoter but elsewhere in the genome. There is also a possibility that population-specific epigenetic mechanisms are taking place.

In a preliminary yeast one-hybrid experiment (Chapter 5) several potential transcription factor binding sites of the \textit{O. cincta} metallothionein promoter were isolated from a cDNA library. This technique implies the screening of interactions between expressed proteins of cDNA clones with bait plasmids (containing potential binding sites) in yeast.

Unfortunately this experiment did not allow us to get a better mechanistic insight in the transcriptional regulation at the metallothionein promoter. However, three potential transcription factors (homologous to respectively CCR4-NOT transcription
complex subunit 3, Y-box protein and *defective proventriculus*) were isolated, which deserve attention in future research.

In Chapter 6 the transcription of approximately 1900 genes was compared between a laboratory culture of reference and a cadmium tolerant population with a transcriptomics approach (microarray analysis). Both control and cadmium-induced expression levels were included in this study. Upon cadmium exposure, the tolerant population showed a less perturbed expression profile compared to the reference population. In 391 genes, a different effect by cadmium exposure was observed between the two populations. Based on their transcription levels, these genes could be grouped in two clusters. The genes from the first clusters exhibited elevated mRNA levels upon cadmium exposure in the reference population. These genes are constitutively highly expressed in the tolerant populations and are involved in the structure of the cuticle, anti-microbial defence, calcium-channel (uptake route of cadmium) blocking, neurotransmitter transport, chromatin remodelling and the endoplasmic vesicle activity. The genes from the second cluster of the cadmium by origin interaction are upregulated upon cadmium exposure in the tolerant population, but not in the reference. They are involved in carbohydrate metabolism, digestion and cellular stress signalling.

In this thesis evidence emerged for the contribution of at least hundreds of genes to the cadmium tolerant phenotype in *O. cincta*. Also the importance of transcriptional regulation for the evolution of this phenotype is demonstrated. New research paths and mechanisms emerged and are awaiting continuing research efforts. In the framework of the regulation at the metallothionein promoter, the isolation of the transcription factors is a prerequisite which should be followed up by binding assays and analysis of the physical status of the chromatin at the *pmt* locus. Mapping of polymorphisms with the cadmium-tolerant phenotype and the metallothionein over-expression, by means of a QTL (quantitative trait loci) method, is obvious. All in all, the role of *cis*-regulation in the transcription of metallothionein is understood better, but the trans-regulatory mechanisms are far from clear.
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Nawoord

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