DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Heritability and inheritance of tolerance to copper in *Daphnia magna*.

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica do Professor Doutor Rui Ribeiro (Universidade de Coimbra) e da Doutora Matilde Moreira-Santos (Universidade de Coimbra)

Filipe José Afonso Sabino de Sousa Louro

2013

Acknowledgements

The work here presented would not have been accomplished without the support of some people and institutions, whom I would like to acknowledge.

Thanks are due to the Fundação para a Ciência e Tecnologia (FCT), Portugal, for partially funding this research project under the project PTDC/BIA-BIC/3488/2012

This work was developed in Instituto do Mar (IMAR) – Department of Life Sciences of the University of Coimbra.

I would like to thank Professor Rui Ribeiro for his supervision and words of encouragement and to Dr. Matilde Moreira-Santos for her orientation and help on the daily work. For their time and patience, for their help with the manuscript but mostly for the opportunity they provided, I am grateful.

To Dr. Luc de Meester I thank the ephippia he kindly provided, for those were crucial for this work.

I would like to acknowledge all the people in IMAR, especially to Rita, Vânia and Tânia for their help and companionship, and all my colleagues in the Masters in Ecology that shared many roads of this path.

I would like to thank to all my friends and family for the support they gave, even not having me near.

To Sara, for being my constant, my better half and for being able to make me smile even after the worst of days, I am thankful.

To my baby sister, I thank the way she is always reminds me to take it easy, and for the smells she brought from home, when home was too far away.

And finally, to my parents, I am grateful for their friendship, for their affection and for being my guardian angels throughout all my life.

Abstract

Genetic erosion is a case of microevolution where genetic information is lost through the loss of genotypes. Although there are many factors that may contribute to the loss of genotypes, contaminants originating from human activities such as mining and agriculture are among the most concerning.

Acid mine drainage (AMD) is a product of mining activity that is a very acidic effluent with high concentrations of various metals, contamination which may lead a population to genetic erosion. In *Daphnia* sp., tolerance to copper was found to be correlated with tolerance to AMD and can be used to study AMD driven genetic erosion. To better understand it two specific objectives were delineated with the present work, to calculate the heritability and inheritance of the tolerance to copper in *Daphnia magna.* Ephippia of *D. magna* were obtained from the following two different sources: (1) a commercial source producing ephippia from uni-clonal mass cultures and (2) a natural pond where ephippia were collected once in time.

Heritability of the tolerance to copper was studied, based on time to death toxicity tests on over 200 clones from two different sources, where values of 71 and 82% were calculated for clones from source M and B, respectively. Relative spread was also calculated for each clone and compared with the tolerance of the respective clones, showing that more extreme clones (the most tolerant and the most sensitive) have smaller variabilities.

To assess inheritance, sexual reproduction was attempted by stimulating ephippia production within a group of 24 selected clones, 12 clones from each source, being the six most sensitive and the six most tolerant to copper. However,

ephippia production was not successfully achieved. Therefore it was not possible to study the inheritance of tolerance to copper in *D. magna*.

Overall, the wide distribution of tolerances, the lower variability in extreme clones, and the high heritability show that tolerance to copper can easily be involved in microevolution episodes. Although not all objectives proposed were achieved, this work still represents new information and one significant contribution to the study of the microevolution of tolerance in *Daphnia magna*.

Resumo

A erosão genética é um caso de microevolução em que a informação genética é perdida através da perda de genótipos. Apesar de haverem muitos fatores que podem contribuir para a perda de genótipos, contaminantes oriundos de atividades humanas como a indústria mineira e a agricultura estão entre os mais preocupantes.

A drenagem ácida de minas (AMD) é um produto da atividade mineira que é um efluente acídico com altas concentrações de vários metais, contaminação esta que pode levar à erosão genética de populações. Em *Daphnia* sp., foi descoberto que a tolerância ao cobre está correlacionada com a tolerância à AMD e pode ser utilizada para estudar a erosão genética provocada pela AMD. Para melhor perceber dois objetivos específicos foram delineados no presente trabalho, calcular a heritabilidade e a herança da tolerância ao cobre em *Daphnia magna.* Ephippia de *D. magna* foram obtidos de duas fontes diferentes: (1) uma fonte comercial que produz ephippia apartir de culturas de massa uni-clonais e (2) um lago natural onde ephippia foram colhidas de uma única vez.

A heritabilidade da tolerância ao cobre foi estudada, baseada em testes de toxicidade de tempo até à morte em mais de 200 clones de duas fontes diferentes, onde foram obtidos valores de 71 e 82% de heritabilidade para os clones oriundos da fonte M e B, respetivamente. O *relative spread* foi também calculado para cada clone e comparado com a tolerância do respetivo clone, mostrando que os clones mais extremos (os mais tolerantes e os mais sensíveis) têm variabilidades menores.

Para avaliar a herança, a indução da reprodução sexuada foi tentada através da estimulação da produção de ephippia num grupo de 24 clones selecionados, 12 clones de cada fonte, sendo os 6 mais sensíveis e os 6 mais tolerantes ao cobre. No entanto, não tenha sido conseguida qualquer produção de ephippia. Assim, não foi possível estudar a herança da tolerância ao cobre em *D. magna*.

Em suma, a larga distribuição de tolerâncias, as variabilidades baixas em clones extremos e a alta heritabilidade mostram que a tolerância ao cobre pode facilmente ser envolvida em episódios de microevolução. Apesar de em todos os objetivos propostos terem sido alcançados, este trabalho representa nova informação e uma contribuição significante para o estudo da microevolução da tolerância em *Daphnia magna*.

Contents

Chapter 1 Introduction

1.1 – The Genetic Erosion Hypothesis

Genetic erosion is a case of microevolution where genetic information is lost through the loss of genotypes (Ouborg et al., 1991; van Straalen and Timmermans, 2002). This process, may lead to the reduced fitness of a population and increase the chance of extinction (van Treuren et al., 1991). Although the causes may differ with the type of population under study, in small populations and populations with little anthropological disturbance, genetic drift and inbreeding are the most usual causes leading to genetic erosion (van Treuren et al., 1993), even though any violation to the Hardy-Weinberg theorem may possibly generate microevolution episodes (Hall and Hallgrimsson, 2011; Ribeiro and Lopes, 2013).

Contaminants may affect the genetic pool by violating each of the five assumptions of the Hardy-Weinberg theorem: (1) Mutagenesis – contaminants may be mutagenic when in sub-lethal doses or even increase the established mutation rates with stress; (2) Genetic Drift – the random annihilation of a significant fraction of a population may change the established genetic frequencies, and even, in some extreme cases, wipe out some alleles, leading the population to a contaminant driven bottleneck; (3) Loss of Panmixis – contaminants may disrupt the randomness of mating by either interfering with the usual behaviour of individuals (e.g. by affecting chemical signalling) or fragmenting the habitat; (4) Emigration – the disruption of one of the directions of the gene flow and/or the aggravation of one through avoidance from contamination may change the genetic frequencies of the population; (5) Natural Selection – contaminants may act as a selection pressure, only allowing the more tolerant individuals to reproduce, either through killing or incapacitating the most sensitive individuals (Belfiore and Anderson, 2001; Ribeiro and Lopes, 2013; Ridley, 1997). However, the most frequent ways for contamination associated with human activities, such as agriculture or mining to affect genetic diversity of natural populations are: (1) isolating a population; (2) reducing the habitat carrying capacity for the population (leading to population bottleneck); (3) reducing the reproductive rate; and (4) increasing mutation rates (Carvajal-Rodríguez et al., 2005). All these could be able to generate genetic erosion in a short term, except for the increase of mutation rates that could only do it in a longer term, if the new mutations would be favourable and result in contaminant driven directional selection, resulting in the consequent loss of the least fit alleles (Ribeiro and Lopes, 2013).

To better understand and study a possible practical case of genetic erosion, heritability and inheritance of the trait being considered should also be studied.

1.2 – Heritability of Tolerance

The distribution of the tolerance to a contaminant in a population is dependent on various factors, being that some of them could be genetic. Genetically determined tolerance to metals is a key factor in ecotoxicological studies, although it is not usually taken into account (Vidal and Horne, 2003). Since the different organisms of a population could show a different tolerance level to each of the possible contaminants, the study of the distribution of the tolerance characters should be taken in mind when, e.g., *in situ* experiments are analysed (Vidal, 2001).

If the tolerance is heritable it can be due to the genetic information or to epigenetic changes. Epigenetic changes are the reversible modifications to the expression of the genome that do not involve mutations and are usually associated with environmental factors. These changes can be reversed within a lifetime or be passed on through some generations, depending on the species and the type of change (LeBaron et al., 2010; Tammen et al., 2013; Vandegehuchte et al., 2010).

Heritability is the proportion of the phenotypic variance of a trait accounted for by genetic variance and can be considered broad-sense (H^2) or narrow-sense (h^2), being that the first considers the variance associated with all the genetic contributions to the phenotype, whereas the latter only considers the variance explained by additive genetic effects (Hoffmann and Merilä, 1999). In this way, heritability may be used as an indication of how genetic factors affect the trait and it is an important indicator of the way the trait may evolve (Hall and Hallgrimsson, 2011). Heritability varies between 0 and 100%, with 0% being that no genetic effects influence the phenotype and 100% that only genetic factors affect the phenotype; it is not a constant since the effects of the environment may fluctuate with space and time though should remain stable under constant environmental conditions if the individuals are acclimated (Barata et al., 2002).

1.3 – Inheritance of Tolerance

The inheritance of tolerance can effectively be split in two categories; Monogenic Inheritance and Polygenic Inheritance (Lande, 1981; Macnair, 1991).

In the first category, the tolerance is inherited from single major genes. This should only occur when a very strong selection is present and a major gene was already moderately frequent before the stress began affecting the population. Such tolerance should remain constant across environments (i.e., the allele responsible for the tolerant phenotype should provide a general form of tolerance, like a thicker carapace that would lower the rate of absorption) (Macnair, 1991).

On the other hand, if the tolerance has a polygenic inheritance, the genetic factors affecting the trait will vary with the environment, since the smaller adaptations will be more specific to each pollutant (Barata et al., 1998). In the majority of the documented cases of evolved tolerance, the latter is endowed by a single major gene. For instance, when *Chironomus riparius* Meigen (and other dipterans) tolerant to metal pollutant are crossed with susceptible ones, the F1 generation shows almost no signs of adaptation to the pollutants (Groenendijk et al., 2002), meaning that the tolerance is a recessive characteristic. Another example is the tolerance to mercury of the aquatic oligochaete *Tubifex tubifex* Mueller, for which study results until the F1 generation show that the inheritance has a dominant nature, but the number of loci or other characteristics could not be assessed (Vidal and Horne, 2003). However, when a population is under stress, recurrent selection may rise the tolerance of the population due to several additive minor genes (Busi and Powles, 2009; Lande, 1981), making the tolerance a continuous characteristic that is controlled by a great number of small features. In these cases, the tolerance is often described as dominant and the heterozygous individuals as having an intermediate response (Groenendijk et al., 2002). These are the most significant cases in studies of macroevolution since artificial selection experiments show that quantitative traits of populations can evolve far beyond the limits of variation in the original population (Lande, 1981).

According to classic Mendelian Genetics, if a certain trait is determined by one single gene with two alleles, said trait may be: (1) Dominant – the trait is totally present in the phenotype whether the individual is homozygous or heterozygous for that allele, (2) Recessive – the individual shows the trait only if it is homozygous for that allele; (3) Incomplete Dominant or Incomplete Recessive– the homozygous for the allele possesses the trait but the heterozygous only show an intermediate form of said trait, according to the intensity of the intermediate form of the trait in heterozygous individuals, it can be incomplete dominant, incomplete recessive or no dominant; (4) Overdominant – the trait is only present in the heterozygous; or (5) Underdominant – the trait is present in both homozygous but not in the heterozygous (Griffiths et al., 2007; Ribeiro and Lopes, 2013). However, nowadays the more popular way for determining the Degree of Dominance is according to the formula developed by Falconer (Stone, 1968) for the cases of monofactorial inheritance of tolerance to chemicals. If after the application of the formula the result is $D = -1$, the heterozygous phenotype is the same as the sensitive homozygous. On the other hand, if the result is $D = 1$ the phenotype of the heterozygous is the same as the tolerant homozygous. Which means that $-1 < D < 0$ corresponds to Incomplete recessivity and $0 < D < 1$ to Incomplete dominance. If $D=0$, the heterozygous individuals have a tolerance equidistant from both homozygous.

1.4 – The Recessive Tolerance Inheritance Hypothesis

When contamination events result in the loss of individuals through death, genetic erosion might be directly associated to the trait of tolerance. Since tolerance to a certain contaminant is usually a genetically determined trait, it is safe to assume that the most sensitive individuals should be the first ones to perish. If in diploid species tolerance can be considered as a trait bestowed by a single gene with two possible alleles, a few possibilities would arise: (1) if tolerance is determined by overdominance or underdominance, the loss of the most sensitive individuals would never result in the loss of either genotype or phenotype, since new sensitive individuals would appear in the next generation, after sexual reproduction; (2) if tolerance is set by dominance, the loss of sensitive individuals would leave the tolerant individuals, both the homozygous and the heterozygous; hence, the recessive phenotype would eventually be restored through the sexual reproduction of two heterozygous individuals; and (3) in the worst case scenario, if tolerance is a recessive trait, upon the loss of all the sensitive individuals the allele to sensitivity would be irreversibly lost, unless it is restored through immigration or a new mutation equal to the one lost (Ribeiro and Lopes, 2013).

In the case of recessive tolerance, allele fixation would occur impacting on biological diversity, which could be aggravated if we take into consideration other hypotheses proposed by Ribeiro and Lopes (2013). Considering that tolerance to a contaminant may have an associated fitness cost to ensure e. g. a faster detoxification, it must be assumed that the individuals, after the allele fixation would not be as fit to populate a restored habitat as others would be. In this case, because of the allocation of resources to tolerate the contamination in detriment of other traits the resilience of this population could be really low. This scenario could even mean that the population, in a contaminant free environment, could find competition e. g. through the introduction of a different population occupying the niche left by the reduction in size of the original populationand be forced to leave or become extinct Another possible aggravator would be the appearance of a new different contamination, since, most likely, the tolerance pathway to the new stressor would be different than the one to the old one, aggravating the already harsh situation of this population.

1.5 – S. Domingos Mine

The S. Domingos mine is an abandoned cupric pyrite mine that has been the target of continuous evolutionary and ecotoxicological studies, motivated by the continuous oxidation of the mine tailings, especially with populations of planktonic cladocerans. After rain events, the oxidation of the pyrite rich sediment produces Acid Mine Drainage (AMD), a very acidic effluent with high concentrations of various metals. This effluent is discharged into the Chança River Reservoir, an otherwise uncontaminated system conditioning the existing aquatic communities (Lopes et al., 1999; Martins et al., 2009). Metals may be toxic to a wide group of organisms, affecting the cell membrane, the genetic material, the nervous system, some enzymes and even causing oxidative damage (Stohs and Bagchi, 1995). These effects can be opposed by a great number of different mechanisms, such as the production of protheins that capture metal ions like those of the family of the metallothioneins and the glutathione, or the accumulation of the metals by mineralization in specific regions (Fraysse et al., 2006; Lopes et al., 2005).

The hypothesis that genetic diversity loss may be significant on this aquatic system has been explored, as contaminant driven genetic erosion of an endemic zooplankton population of *Daphnia longispina* O.F. Müller has been extensively tested (Lopes et al., 2006; Martins et al., 2009; Ribeiro et al., 2012). Even though AMD has been showed to have mutagenic capabilities at sub-lethal concentrations (Sobral et al., 2013), if existent, genetic erosion in the latter population in São Domingos mine would mostly be resultant of the loss of the individuals less tolerant to the AMD contamination,

1.6 – Study Objectives

To better understand the possibility of the loss of genetic information due to contamination through genetic erosion, a study organism was needed that enabled the study of the heritability and inheritance of the tolerance. *D. magna* Straus was chosen for it is a frequently used cladoceran for ecotoxicity assays, mostly because of the advantages they proportionate. They are easy to handle, have a rather short lifespan and a high sensitivity to contaminants (Barata et al., 1998; Miranda et al., 2011; Pereira et al., 2007). Besides that, they reproduce asexually through cyclical parthenogenesis, which means that each female gives origin to broods of females genetically identical to it (Slack, 2005).

Since AMD is a complex solution with many possibly toxic components (with a low pH), it would be very hard to determine heritability and inheritance of its tolerance, since there may be many mechanisms conferring tolerance to the different components. Therefore, tolerance to copper will be determined instead, once it is among the metals in higher concentration in AMD and a strong correlation between the tolerance to lethal levels of copper and AMD was previously reported to a *Daphnia* species (Martins et al., 2007)

Therefore, with the present work, it was intended to study the tolerance of *D. magna* to copper. For this, two specific objectives were delineated, to calculate the Heritability and Inheritance of the tolerance to copper in *D. magna.*

To study heritability, a large number of clones of *D. magna* was characterized according to their tolerance to copper. To do this, Time To Death (TTD) toxicity tests were performed. Then, variances within and among clones were analyzed to calculate heritability,

To study inheritance, the more extreme clones (the more sensitive and the more tolerant) will be chosen to produce ephippia through sexual reproduction from each of the extreme clones. TTD toxicity tests should be performed with the new clones hatched from the ephippia. The pattern of tolerances of the new clones should be analyzed to assess the ratios of tolerance and to understand how tolerance is passed on through generations.

With the present work, it is hoped that new light will be shared on the microevolution of copper tolerance in *D. magna* and contribute to the study of AMD contamination driven genetic erosion.

Chapter 2 Materials and Methods

2.1- *Daphnia magna*

D. magna Strauss, 1820 is a freshwater cladoceran, usually considered to be an important constituent of zooplankton in many lentic systems, and the biggest species of the *Daphnia* genus (Adema, 1978; Moreira dos Santos, 1998). Organisms of this genus are non-selective filter feeders and ingest usually unicellular algae (Barnes, 1987). They have a lifespan that can go up to two months and can reproduce either sexually or asexually, through cyclic parthenogenesis (Ebert, 2005; Saro et al., 2012; Slack, 2005). When reproducing asexually by parthenogenesis under optimal laboratory conditions (mainly of temperature and food), the female *D. magna* gives birth to the first brood of genetically identical neonates (10 to 18 neonates) between the 7th and the 9th day after they were born and then begins to release one brood (of 20 to 40 neonates) every 3 days (Ebert, 2005). Under unfavourable conditions sexual reproduction will usually take place (Moreira dos Santos, 1998). Females are able to produce haploid males through parthenogenesis that will fertilize a maximum of two eggs released into the brood pouch; these eggs, fertilized or not, are laid enclosed in a shell called ephippium, capable of resisting harsh conditions and neonates hatch when optimal conditions are again present (Moreira dos Santos, 1998). Sexual reproduction is the only way for *Daphnia* sp. to achieve genetic recombination.

2.2- Ephippia origin and neonate hatching

Ephippia of *D. magna* were obtained from the following two different sources: (1) a commercial source producing ephippia from uni-clonal mass cultures (MicroBioTests, Mariakerke, Belgium) (2) a natural pond (Langerodevijver; Leuven, Belgium) where ephippia were collected once in time and gently provided by Luc de Meester (KU Leuven, Belgium). For neonate hatching, ephippia were incubated in reconstituted hard water *Daphnia* sp. culture medium from the American Society for Testing and Materials (ASTM, 2002), hereafter called ASTM medium, at 20-22 ºC under continuous illumination of approximately 6,000 lux (Microbiotests). The vast majority of the neonates hatched after an incubation period of 72 hours, time after which they were immediately collected and individually cultured.

2.3- Cultures of *Daphnia magna* clones

After hatching, neonates were isolated and cultured individually, meaning that from here onwards, each isolated neonate and its progeny are considered genetically identical and, therefore, a clone. Clones descendant from different sources were separated into two groups. The clones originated from the commercial source shall be called henceforth the group of clones M and the clones from the natural pond will be called the group of clones B. Individual cultures consisted of 40 or 50 ml glass vials with ASTM medium enriched with vitamins (7.5 µg/l of B_1, 1 µg/l of B_{12} and 0.75 µg/l of biotin) and seaweed extract (“Marinure” at 7.5 ml/l of a suspension with an absorbance of 620 units at 400 nm; Glenside, Stirling, UK). Organisms were fed daily with $3x10^5$ cells/ml of the microalgae *Pseudokirchneriella subcapitata* (Koršhikov) and culture medium was changed every other day. Up to 7 days old, crystalized cetyl alcohol (Panreac Quimica, Barcelona, Spain) was added to the medium to decrease surface water tension and avoid buoyancy of the small daphnids (Desmarais, 1997). All cultures were kept under a 16:8 L:D photoperiod at 19-21 ºC. Each new generation was initiated with a single descendant between the third and the fifth broods.

2.4- Tolerance to copper characterization (TTD toxicity tests)

The tolerance of each clone to the model toxicant copper was determined through Time To Death (TTD) toxicity tests. The latter were performed with neonates less than 24-hours old released between the third and fifth broods after at least three generations after hatching, to ensure acclimation. At the start of the test, neonates were individually transferred to glass vials with 40 ml of non-enriched (see above) ASTM medium with a copper concentration of 0.35 mg/l since this concentration was found to be suitable to cause a gradient of an increase in mortality (from 0 to 100%) with increasing exposure time after preliminary testing. No food was provided during testing but cetyl alcohol surfactant addition and incubation conditions were as the stock cultures. To prepare the copper test solutions, a stock solution of 100 mg/l of copper was prepared with $CuSO_4.5H_2O$ (Merck, Darmstadt, Germany) in nanopure water and stored at 4 ºC in darkness for a maximum period of one month.

During testing, individuals were observed at the following increasing intervals of exposure time: 0.5, 1.0, 1.8, 3.2, 5.6, 10.0, 17.8, 31.6, 56.2, and 100 hours. Individuals were considered dead when immobilized for more than 15 seconds after gentle agitation of the container, as recommended in standard guidelines for lethal toxicity tests with *Daphnia* sp. (OECD, 1984). Between 20 and 64 replicates were performed for each clone with 10 to 32 control individuals cultured solely in ASTM medium; though not all replicates were performed once in time, at each point in time both control and copper contaminated replicates were set up with the number of control individuals being at least half the number of replicates of each clone. For each test to be valid, no more than 10% of the control individuals should be considered dead (OECD, 1984). These TTD toxicity tests were performed with 105 clones from group M and 101 clones from group B.

2.5- Production of ephippia

For each group of clones descendant from the two different ephippium sources, ephippium production by the six most sensitive and six most tolerant clones (i.e., a total of 24 clones) was attempted by exposing female *Daphnia* to two unfavourable stimuli, an increase in population density at constant food levels followed by a consequent increase in food shortage (Moreira dos Santos, 1998). This was achieved by starting cultures with neonates less than 24-hours old (from third to fifth broods) at two different population densities, 200 and 400 organisms/l, in either 50 or 100 ml cultures. These cultures were kept exactly under the same conditions as the stock cultures, except for the two following conditions: (1) the food quantity daily given was doubled and (2) the population density was not kept at constant levels since newborns were not retrieved from the cultures, which led to a decrease in food availability per organism. The starting low levels of stress should induce the production of haploid males through parthenogenesis after a few weeks and, then, at the increasing stress level, the production of haploid eggs and their fertilization by the mature males would lead to the production of ephippia that should be laid by female *D. magna* approximately six weeks after the initial exposure to the stressor.

2.6- Data analysis

Given that the production of ephippia by the clones with extreme sensitivities could not be achieved in the present study, only the data analysis regarding the study of heritability (variability within and among clones) will be taken in account.

2.6.1 – Heritability of tolerance

The characterization of intraclonal variation was accomplished through the calculation of two toxicity parameters: (1) the LT_{50} (median lethal time, i.e., time of exposure at which the copper concentration of 350 mg/l is lethal to 50% of the individuals of the clone) and (2) the relative spread of the replicates of each clone (i.e., the response variation of each clone). LT_{50} values were calculated with the software PriProbit v1.63 that analyses the regression between the logarithmic transformation of observation times and the probit transformation of the mortality response of organisms. An All-or-Nothing-Model was applied to calculate the mortality curve for each clone. Each Relative Spread (RS) was calculated as the percentage of the difference between the third (LT_{75}) and first quartiles (LT_{25}) of the distribution of Lethal Time relatively to the LT_{50} value, with the following formula: $RS = (LT_{75} – LT_{25}) \times 100/LT_{50}$.

2.6.2 – Inheritance of tolerance

Regarding interclonal variation, its characterization was done through the calculation of heritability. Although there are two kinds of heritability that can be calculated (broad-sense and narrow-sense), the more appropriate estimation of heritability is the broad-sense heritability (H^2), since *D. magna* reproduces parthenogenetically and narrow-sense heritability would be harder to calculate without sexual recombination (Carter et al., 2012; Stirling and Roff, 2000).

H^2 shows the amount of phenotypical variance (V_P) explained by the genetic information (V_G), i.e., how much do genetic factors influence the trait, being $H^2 = V_G / V_P$.

Since individuals in the same clone have similar genetic information, all the variance between replicates of the same clone is not explained by genetic effects but rather by environmental factors. Consequently, the variance calculated within each clone should be considered environmental variance (V_E). Since phenotypical variance should be the sum of all variances $V_P=V_G+V_E$, V_G can be found by subtracting the environmental variance in the total phenotypical variance, i.e., $V_G=V_P–V_E$. Therefore, H^2 for each group of *D. magna* clones descendant from the two different ephippium sources was calculated with the following formula: $H^2 = (V_P-V_E)/V_P$.

Chapter 3 Results

3.1- Heritability of tolerance

The clones were essayed and the LT_{50} of the time to death in hours was calculated. A histogram was charted, showing the distribution of the LT_{50} in the logarithm scale for a better ecological significance and to facilitate analysis, as seen in figure 1. M clones showed an amplitude of over one magnitude order, from 2.8 to 42.5 hours, and a modal distribution. B clones showed also an amplitude over one magnitude order from 54 minutes to 29.4 hours. These clones were distributed over a non-distinctive pattern.

Relative spreads were also calculated for each clone and compared with the tolerance of the same clone (in LT_{50}) on a logarithmic scale (Figure 2). A distinct pattern was found with the extreme clones (the most sensitive and the

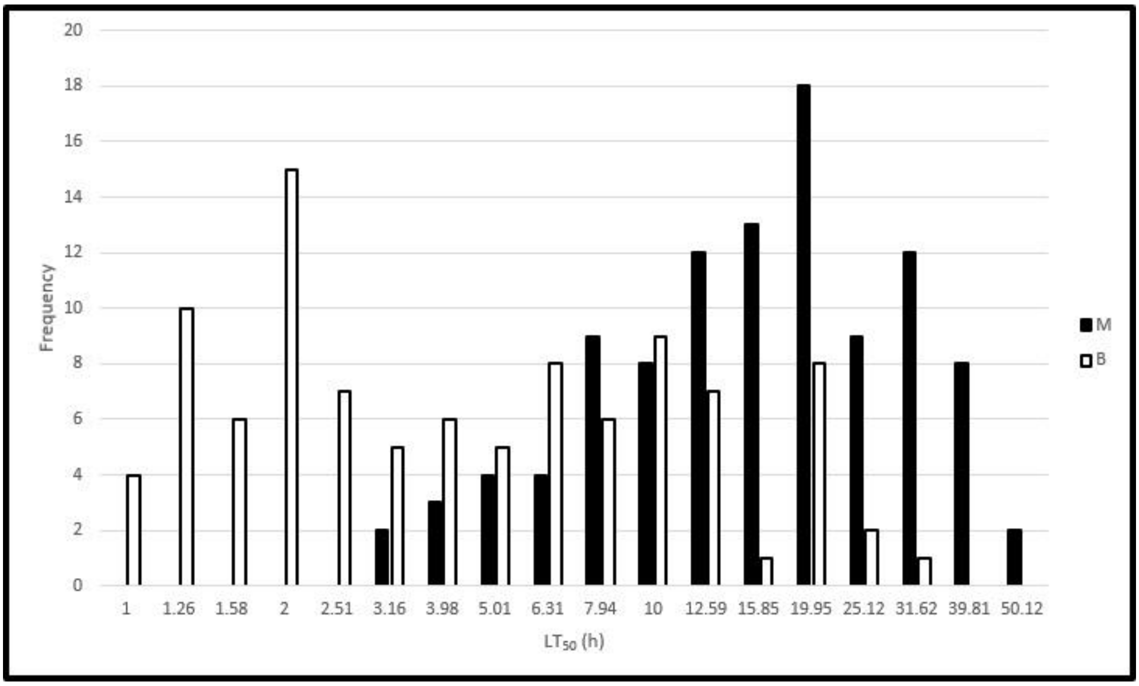

Figure 1 - Distribution of the tolerance to copper of two groups of clones of *Daphnia magna*, clones originated from a commercial source (M) and clones from a natural pond (B), shown as the LT50 of each clone with clones split into categories on a logarithmic scale

most tolerant) showing lower relative spreads. Clones with intermediate tolerance showed a relative spread ranging from 3 to 230%.

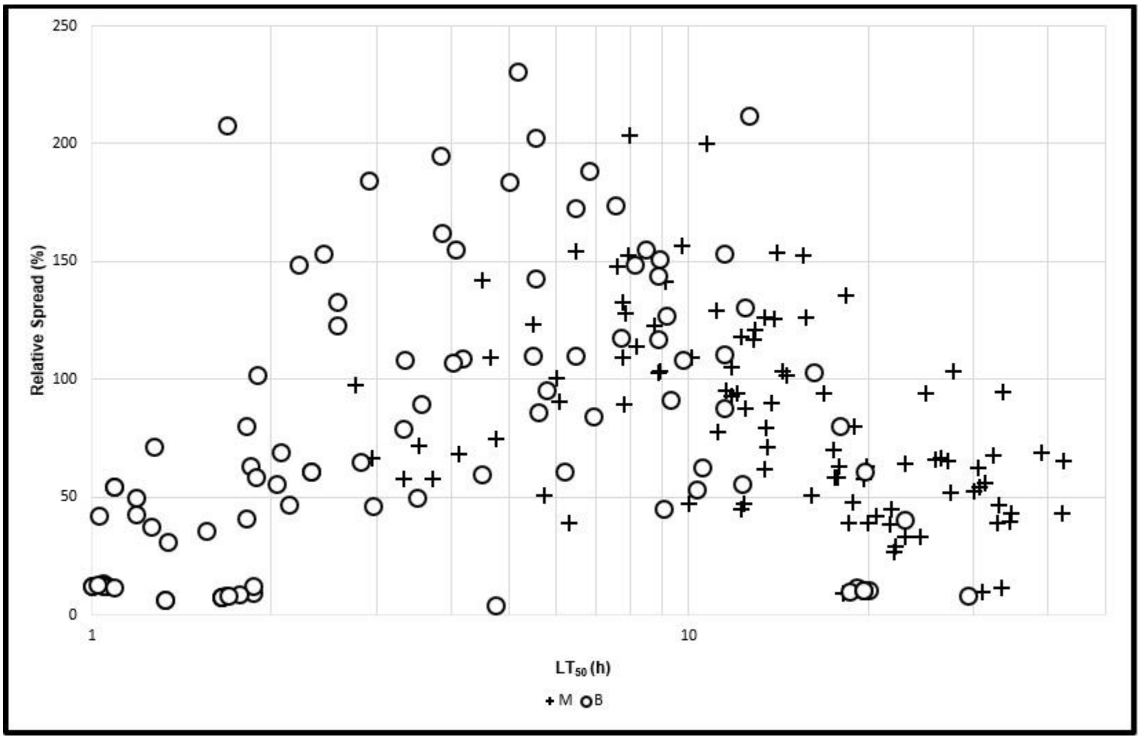

Figure 2 - Variation within *Daphnia magna* clones shown as relative spread (in percentage) in function of the tolerance of the respective clone shown as LT_{50} in hours on a logarithmic scale

Broad-sense heritability was calculated as shown before. Genetic factors explained 71% of the variance in group M clones and 82% in group B, as shown in Table I.

Table I - Calculation of Broad-Sense Heritability in the two groups of *Daphnia magna*, clones from a commercial source (M) and clones from a natural pond (B), showing values of the environmental and phenotypical variances.

Group of Clones	**V_E**	**V_P**	**H^2**	**H^2 (%)**
M	0.522	1.8	0.710	71%
B	0.500	2.76	0.819	81.9%

3.2 – Inheritance of tolerance

Ephippia were expected to be laid by female *D. magna* after approximately six weeks of culturing under the selected unfavorable conditions. However, no ephippium production by any of the 24 clones selected for this purpose was observed after more than eight weeks.

Chapter 4 Discussion

4.1 – Heritability of tolerance

Results from the time to dead toxicity tests expressed as LT_{50} values showed that the clones from group B had a wide range of tolerance with a bimodal distribution, while the clones from group M showed a narrow and normal distribution of tolerance. It was expected for group M clones to have the narrowest range of tolerances, since the ephippia originating these clones were produced in the laboratory and all derived from a uni-clonal mass culture. This should have originated a founder effect, responsible for a low genetic diversity (Ridley, 1997). On the other hand, the clones from the B group were expected to have the greatest tolerance, and thus genetic diversity, since they were collected from the sediment of a natural pond, possibly representing several populations that occupied that habitat over the time.

The distribution without a distinctive pattern found in the tolerance of the clones from group B may be caused by the type of reproduction of *D. magna*. With sexual reproduction, ephippia are produced and laid in the sediment until a stimulus associated with favourable conditions induces the neonates to hatch from ephippia that remained viable for decades (Keller and Spaak, 2004). The stimulus necessary for hatching is different among individuals, and it is very unlikely that all the neonates will hatch from all ephippia. Therefore, it is probable that ephippia found in the sediment are representative of several populations of different times and with different clones (Ebert, 2005).

The relative spread was calculated for the cumulative death of individuals in each clone (time to death toxicity tests). Analyzing the pattern found between the relative spread and the LT_{50} values, higher relative spread values can be found in clones with intermediate tolerance to copper. This can be explained by the fact that for clones to show an extreme tolerance or sensitivity, most replicates from that clone were surely extremely tolerant or sensitive, respectively.

The H^2 values calculated showed that genetic factors provide a very important contribution for the phenotype of the tolerance. The small difference between the two groups of clones may be explained by the relative spread values. Clones of the M group tend to have more tolerant clones and because of the bigger intervals between the last readings, the variance of the individuals inside each clone are higher. This can show as a higher environmental variance and, therefore, a lower heritability.

Overall, the wide distribution of tolerances, the lower variability in extreme clones, and the high heritability show that tolerance to lethal levels of copper is a trait that can easily be involved in microevolution episodes.

4.2 – Inheritance of tolerance

After 8 weeks of stimuli no ephippium was deposited nor was observed in the brood pouch of females, which suggests that the stimuli were not enough to induce the production of male *D. magna* through parthenogenesis or that males were produced but sexual reproduction did not happen.

Literature shows that different stimuli can be used to produce males, being population density and food shortage among the more utilized with photoperiod and temperature, but also that each clone is different and most have different thresholds for each stimulus (Moreira dos Santos, 1998). So, for sexual reproduction to be induced, more attention should be given in future studies to each clone and different types of stimuli and intensities should be tried (Zhang and Baer, 2000).

On the other hand, if males were produced, two explanations may be given to the fact that no ephippia were produced: (1) the stimulus was not enough to lead to sexual reproduction or (2) the stress inflicted was too unfavourable and inhibited sexual reproduction.

Sexual reproduction is also induced by environmental factors like food availability, light intensity and photoperiod among others and, just like in male production, responses vary among and within populations, having a big variability (Moreira dos Santos, 1998). Authors refer yet that *Daphnia* sp. possess impedances against inbreeding and that stimuli needs to be higher to produce intraclonal ephippia (Winsor and Innes, 2002). Still, the environment proportionated could have been too harsh and inhibited the males' disposition for sexual reproduction. As such, new intensities of the stimulus and new stimuli should be tried.

4.3 – Concluding Remarks

Although not all objectives proposed were achieved, this work still represents new information and one significant contribution to the study of the microevolution of tolerance in *Daphnia magna*. Further efforts will be needed in the assessment of inheritance of tolerance to fully complete this area of knowledge.

References

Adema, D.M.M., 1978. Daphnia magna as a test animal in acute and chronical toxicity tests. Hydrobiologia 59, 125-134.

ASTM (American Society for Testing and Materials), 2002. Standard guide for conducting acute toxicty tests on tests on test materials with fises, microinvertebrates, and amphibians, in: ASTM (Ed.), Annual Book of America Society of Testng and Materials Standards, Philadelphia, PA, USA, pp. 729-796.

Barata, C., Baird, D.J., Markich, S.J., 1998. Influence of genetic and environmental factors on the tolerance of Daphnia magna Straus to essential and non-essential metals. Aquatic Toxicology 42, 115-137.

Barata, C., Markich, S.J., Baird, D.J., Taylor, G., Soares, A.M.V.M., 2002. Genetic variability in sublethal tolerance to mixtures of cadmium and zinc in clones of Daphnia magna Straus. Aquatic Toxicology 60, 85-99.

Barnes, R.D., 1987. Invertebrate zoology. Saunders College Pub.

Belfiore, N.M., Anderson, S.L., 2001. Effects of contaminants on genetic patterns in aquatic organisms: a review. Mutation Research/Reviews in Mutation Research 489, 97-122.

Busi, R., Powles, S.B., 2009. Evolution of glyphosate resistance in a Lolium rigidum population by glyphosate selection at sublethal doses. Heredity 103, 318-325.

Carter, M.J., Simon, J.C., Nespolo, R.F., 2012. The effects of reproductive specialization on energy costs and fitness genetic variances in cyclical and obligate parthenogenetic aphids. Ecology and Evoluton 2, 1414-1425.

Carvajal-Rodríguez, A., Rolán-Alvarez, E., Caballero, A., 2005. Quantitative variation as a tool for detecting human-induced impacts on genetic diversity. Biological Conservation 124, 1-13.

Desmarais, K.H., 1997. Keeping Daphnia out of the surface film with cetyl alcohol. Journal of Plankton Research 19, 149-154.

Ebert, D., 2005. Chapter 2, Introduction to Daphnia Biology, Ecology, Epidemiology, and Evolution of Parasitism in Daphnia. Bethesda (MD): National Center for Biotechnology Information (US), National Center for Biotechnology Information (US).

Fraysse, B., Geffard, O., Berthet, B., Quéau, H., Biagianti-Risbourg, S., Geffard, A., 2006. Importance of metallothioneins in the cadmium detoxification process in Daphnia magna. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 144, 286-293.

Griffiths, A.J.F., Wessler, S.R., Lewontin, R.C., Gelbart, W.M., Suzuki, D.T., 2007. Introduction to Genetic Analysis, 8th ed. Macmillan Higher Education.

Groenendijk, D., Lucker, S.M.G., Plans, M., Kraak, M.H.S., Admiraal, W., 2002. Dynamics of metal adaptation in riverine chironomids. Environmental Pollution 117, 101-109.

Hall, B.K., Hallgrimsson, B., 2011. Strickberger's Evolution, 4th ed. Jones & Bartlett Learning.

Hoffmann, A.A., Merilä, J., 1999. Heritable variation and evolution under favourable and unfavourable conditions. Trends in Ecology & Evolution 14, 96-101.

Keller, B., Spaak, P., 2004. Nonrandom sexual reproduction and diapausing egg production in a Daphnia hybrid species complex. Limnology and Oceanography 49, 1393-1400.

Lande, R., 1981. The minimum number of genes contributin to quantitative variation between and within populations. Genetics 99, 541-553.

LeBaron, M.J., Rasoulpour, R.J., Klapacz, J., Ellis-Hutchings, R.G., Hollnagel, H.M., Gollapudi, B.B., 2010. Epigenetics and chemical safety assessment. Mutation Research/Reviews in Mutation Research 705, 83-95.

Lopes, I., Baird, D.J., Ribeiro, R., 2005. Resistance to metal contamination by historically-stressed populations of Ceriodaphnia pulchella: Environmental influence versus genetic determination. Chemosphere 61, 1189-1197.

Lopes, I., Baird, D.J., Ribeiro, R., 2006. Genetic adaptation to metal stress by natural populations of Daphnia longispina. Ecotoxicology and Environmental Safety 63, 275-285.

Lopes, I., Gonçalves, F., Soares, A.M.V.M., Ribeiro, R., 1999. Discriminating the Ecotoxicity due to Metals and to Low pH in Acid Mine Drainage. Ecotoxicology and Environmental Safety 44, 207-214.

Macnair, M.R., 1991. Why the evolution of resistance to anthropogenic toxins normally involves major gene changes – the limits to natural-selection. Genetica 84, 213-219.

Martins, N., Bollinger, C., Harper, R.M., Ribeiro, R., 2009. Effects of acid mine drainage on the genetic diversity and structure of a natural population of Daphnia longispina. Aquatic Toxicology 92, 104-112.

Martins, N., Lopes, I., Harper, R.M., Ross, P., Ribeiro, R., 2007. Differential resistance to copper and mine drainage in Daphnia longispina: Relationship with allozyme genotypes. Environmental Toxicology and Chemistry 26, 1904-1909.

Microbiotests, Hatching of the Ephippia, in: Inc., M. (Ed.), Crustacean Toxicity Screening Test for Freshwater - Standard Operational Procedure, Mariakerke (Gent), Belgium.

Miranda, A.F.P., Rodrigues, J.M.L., Barata, C., Riva, C., Nugegoda, D., Soares, A., 2011. The use of Daphnia magna immobilization tests and soil microcosms to evaluate the toxicity of dredged sediments. Journal of Soils and Sediments 11, 373-381.

Moreira dos Santos, M., 1998. The Controled Production and Hatching of Ephippia of Daphnia magna (Crustacea: Cladocera) for Toxicity Testing. University of Gent, Gent.

OECD (Organisation for Economic Co-operation and Development), 1984. Daphnia sp. Acute Immobilization Test and Reproduction Test, in: OECD (Ed.), OECD Guideline for Testing of Chemicals.

Ouborg, N.J., van Treuren, R., van Damme, J.M.M., 1991. The significance of genetic erosion in the process of extinction II. Morphological variation and fitness components in populations of varying size of Salvia pratensis L. and Scabiosa columbaria L. . Oecologia 86, 359-367.

Pereira, J.L., Mendes, C.D., Gonçalves, F., 2007. Short- and long-term responses of Daphnia spp. to propanil exposures in distinct food supply scenarios. Ecotoxicology and Environmental Safety 68, 386-396.

Ribeiro, R., Baird, D.J., Soares, A.M.V.M., Lopes, I., 2012. Contaminant driven genetic erosion: A case study with Daphnia longispina. Environmental Toxicology and Chemistry 31, 977-982.

Ribeiro, R., Lopes, I., 2013. Contaminant driven genetic erosion and associated hypotheses on alleles loss, reduced population growth rate and increased susceptibility to future stressors: an essay. Ecotoxicology.

Ridley, M., 1997. Evolution, 2nd ed. Oxford University Press.

Saro, L., Lopes, I., Martins, N., Ribeiro, R., 2012. Testing hypotheses on the resistance to metals by Daphnia longispina: differential acclimation, endpoints association, and fitness costs. Environmental Toxicology and Chemistry 31, 909-915.

Slack, J.M., 2005. Essential Developmental Biology, 2nd ed. Wiley, John & Sons, Incorporated.

Sobral, O., Marin-Morales, M.A., Ribeiro, R., 2013. Could contaminant induced mutations lead to a genetic diversity overestimation? Ecotoxicology (London, England).

Stirling, G., Roff, D.A., 2000. Behaviour plasticity without learning: phenotypic and genetic variation of naïve Daphnia in an ecological trade-off. Animal Behaviour 59, 929-941.

Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radical Biology and Medicine 18, 321-336.

Stone, B.F., 1968. A formula for determining degree of dominance in case of monofactorial inheritance to chemicals. Bull. World Health Organ. 38, 325 - 326.

Tammen, S.A., Friso, S., Choi, S.-W., 2013. Epigenetics: The link between nature and nurture. Molecular Aspects of Medicine 34, 753-764.

van Straalen, N.M., Timmermans, M., 2002. Genetic variation in toxicant-stressed populations: An evaluation of the "genetic erosion" hypothesis. Human and Ecological Risk Assessment 8, 983-1002.

van Treuren, R., Bijlsma, R., Ouborg, N.J., van Delden, W., 1993. The Significance of Genetic Erosion in the Process of Extinction. IV. Inbreeding Depression and Heterosis Effects Caused by Selfing and Outcrossing in Scabiosa columbaria. Evolution 47, 1669-1680.

van Treuren, R., Bijlsma, R., van Delden, W., Ouborg, N.J., 1991. The significance of genetic erosion in the process of extinction. I. Genetic differentiation in Salvia pratensis and Scabiosa columbaria in relation to population size. Heredity 66, 181-189.

Vandegehuchte, M.B., Vandenbrouck, T., De Coninck, D., De Coen, W.M., Janssen, C.R., 2010. Can metal stress induce transferable changes in gene transcription in Daphnia magna? Aquatic Toxicology 97, 188-195.

Vidal, D.E., 2001. Tolerance and Adaptation of Aquatic Oligochaetes to Mercury Contamination. University of California, Berkeley.

Vidal, D.E., Horne, A.J., 2003. Inheritance of mercury tolerance in the aquatic oligochaete Tubifex tubifex. Environmental Toxicology and Chemistry 22, 2130-2135.

Winsor, G.L., Innes, D.J., 2002. Sexual reproduction in Daphnia pulex (Crustacea : Cladocera): observations on male mating behaviour and avoidance of inbreeding. Freshwater Biology 47, 441-450.

Zhang, L., Baer, K.N., 2000. The influence of feeding, photoperiod and selected solvents on the reproductive strategies of the water flea, Daphnia magna. Environmental Pollution 110, 425-430.