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MICRO-EVOLUTIONARY EFFECTS OF Cd ON NATURAL *DAPHNIA MAGNA* POPULATIONS

Thesis submitted in the fulfillment of the requirements
For the degree of Doctor (PhD) in Applied Biological Sciences

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List of abbreviations

ANOVA	Analysis of Variance
ECB	European Chemical Bureau
Cd	Cadmium
Cov	Covariance
CV_A	Additive genetic coefficient of variation
CV_G	Genetic coefficient of variation
DNA	Desoxyribonucleid acid
DOC	Dissolved Organic Carbon
Dry wt	Dry weight
ECHA	European Chemical Agency
ECx	Effect Concentration resulting in x effect
EQS	Environmental Quality Standard
EU	European Union
H^2	Broad sense heritability
h^2	Narrow sense heritability
HC_5	Hazard concentration for 5% effect
LCx	Lethal concentration resulting in x effect
LOEC	Lowest Observed Effect Concentration
NOEC	No Oberved Effect Concentration

OECD	The Organisation for Economic Co-operation and Development
PNEC	Predicted No Effect Concentration
R	Response to selection
RA	Risk Assessment
RAR	Risk Assessment Report
R_0	Total reproduction during 21 days
REML	Restricted maximum likelihood
r_m	Population growth rate
S	Selection differential
US-EPA	US Environmental Protection Agency
V_A	Additive genetic variance
V_D	Dominance genetic variance
V_G	Genetic variance
V_P	Phenotypic variance
WHAM	Windermere Humic Aqueous Model

Chapter 1: Introduction and conceptual framework

1. Conventional ecotoxicology versus evolutionary ecotoxicology

The ultimate goal of environmental risk assessment is to prevent chemical substances causing irreversible damage to ecosystems (e.g. European Commission, 2006). In conventional ecotoxicology, tolerance to stress is tested under standardized laboratory conditions. However, natural field populations are exposed to a mixture of stressors and fluctuations of abiotic exposure conditions. There are several studies that indicate that for example temperature increase (Heugens et al., 2003), food quantity/quality (e.g. Heugens et al., 2006), dissolved oxygen (Ferreira et al., 2010) can have substantial effects on tolerance for toxicants.

Unfortunately, conventional ecotoxicology is too often focused on the short-term effects of stressors (Morgan et al., 2007). By using, for example monoclonal *D. magna* populations or inbred populations, genetic variability is minimized, which increases the precision of the estimation of mean population responses and also decreases the variability among toxicity tests. This increases the repeatability, reproducibility and robustness of toxicity tests (Barata et al., 2000a). Although differences in mean population responses were observed in studies of Barata et al. (2002a,b,c); Lopes et al. (2004,2005,2006); Agra et al. (2010); Coors et al. (2009), the number of studies that have investigated the between-population variability responses to stressors among populations originating from pristine environments are limited. Besides differences in mean population responses, there are numerous studies that have indicated genetic variability within a population in response to stressors. Baird et al. (1990), for example, found a genetic variability in acute Cd tolerance up to a factor 100 between clones. Barata et al. (2002b) observed a significant difference in EC_{50} of feeding rate for three *D. magna* clones ranging between 2.2 µg Cd/L and 15.4 µg

Cd/L. A study performed with 8 *Daphnia magna* clones indicated 48h-EC_{50}'s between 26 µg Cd/L and >120 µg Cd/L (Ward and Robinson, 2005), whereas the 48h-EC_{50}'s from seven *Daphnia magna* clones ranged between 250 µg Cd/L and 550 µg Cd/L in a study from Haap and Köhler (2009). Genetic variability has also been studied for other substances. A 10-fold difference in 48h-EC_{50}'s between *D. magna* clones was observed for effluent samples (Picado et al., 2007). There were also differences in reproduction parameters noted for chronic azoxystrobine exposure between three *D. magna* clones (Warming et al., 2009). Genetic variation in sensitivity to Cd was also observed for *Chironomus riparius* (Nowak et al., 2008), where there was considerable variation in reproduction both in control and under Cd exposures. Jensen and Forbes (2001) found significant difference in LC_{50} values between tree clones of *Potamopyrgus antipodarum*. Crommentuijn et al. (1995) found differences between four clones of *Folsomia candida* for chlorpyrifos in a 35-day artificial soil test, but not for Cd and triphenyltin hydroxide. Another aspect that has received less attention from ecotoxicologists are the changes in this genetic variability and allele frequencies of populations that result from mutations, bottlenecks and selection. The study of such effects has been termed "evolutionary ecotoxicology" (Bickham et al., 2000; Bickham et al., 1994).

Although the use of monoclonal populations guarantees low variability of test results, it is unreliable to predict long-term effects of chemical exposure in the field. Evolutionary changes in populations exposed to polluting events depend on how the toxicants disrupt the genetic pool of the exposed populations. There can be direct effects (see Medina et al., 2007 for detail), which are related to the damage that toxic substances exert on the molecular structure of the genetic code (i.e. DNA) and indirect effects where pollution changes the genetic variability of the population. Examples of direct effects are: point mutation,

chromosomal re-arrangements, inversions, deletions, additions, DNA adducts, DNA strand breaks, excess of micronuclei and mitotic aberrations. If direct effects are exerted on somatic cells, this is not passed on the following generation. In addition, through natural selection, any effects on fitness resulting from such direct effects on somatic cells would be rapidly eliminated. However, when direct effects are exerted on gametes, significant effects on the following generation can occur (see Medina et al., 2007). Indirect effects are population-mediated processes where pollution changes the genetic variability of the population (Medina et al., 2007) and according to Van Straalen and Timmermans (2002), there are four different ways in which toxicants affect genetic variation: (1) by increasing mutation rates, (2) by directional selection on tolerant genotypes, (3) by causing bottleneck events and (4) by altering migration. Figure 1.1 (after Van Straalen and Timmermans, 2002) illustrates a conceptual framework for effects of toxicants on genetic variation in natural populations. A first step is to distinguish between neutral and selectable markers. Neutral markers are all traits that are indifferent to the selection of a specific environment. Selectable markers are traits that directly respond (fitness advantage or disadvantage) to the selection regime. Those types of markers can be linked due to genetic linkage. Several genetic mechanisms may be possible: epistasis (alleles of one locus depend on the presence of alleles at another locus), pleiotropy (one gene product affects more than one phenotypic character), co-adapted gene complexes (certain alleles are jointly beneficial) and trade-offs. The type of selection regime on neutral markers also determines a decreased or increased genetic variation. Decreased genetic variation may be possible through stabilizing selection (i.e. selection against both high and low extremes of a character), whereas increased genetic variation may be possible through disruptive selection (selection favoring both high and low extremes of a character). Mutation, migration (exchange of genotypes) and population size

may also influence the outcome of genetic variation in a population. Genetic variation in small populations is sensitive to the effects of genetic drift (i.e. random changes in allele frequencies due to chance effects from one generation to another). If toxicants cause long-term reduction or fragmentation of populations, genetic variation may decrease without directly selecting the markers under consideration. Population bottleneck is a special case of drift, when population size is significantly reduced (by anthropogenic or natural stressors), leaving only a small population as a founder for recovery and expansion (see Van Straalen and Timmermans, 2002).

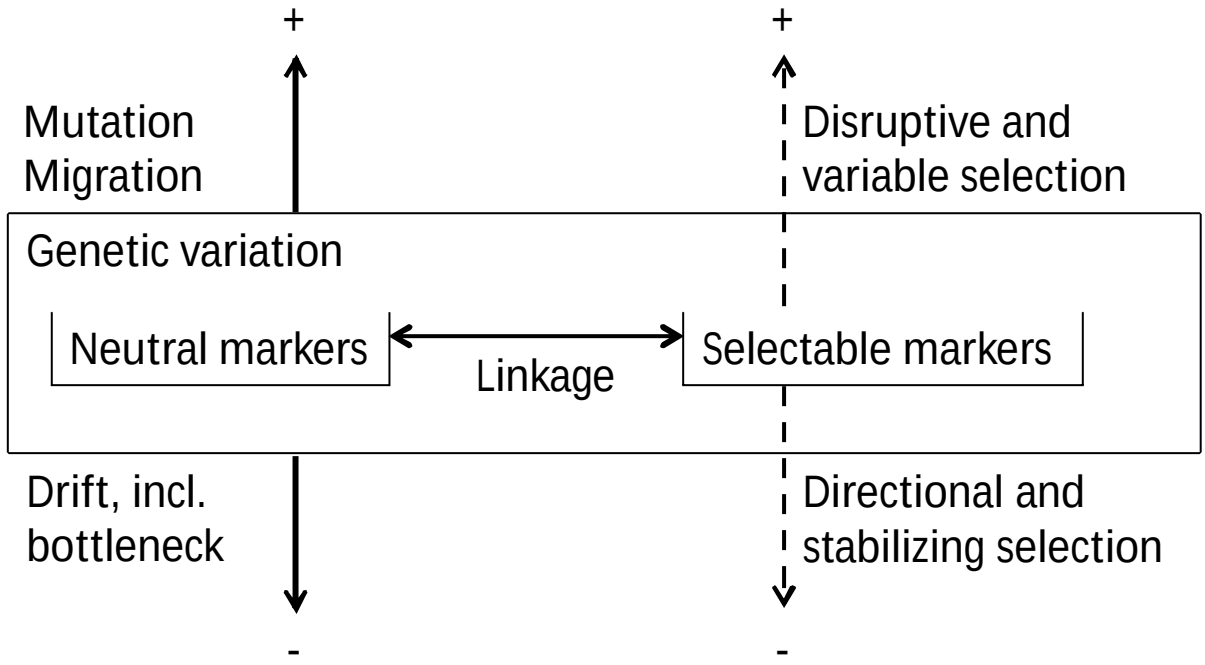

Figure 1.1: A Conceptual framework for effects of toxicants on genetic variation in natural populations (After Van Straalen and Timmermans, 2002). + indicates an increase in genetic variation, - indicates a decrease in genetic variation. Factors related to population size (drift, bottleneck), mutation and immigration will affect neutral and selectable markers. Selection only works on selectable markers. Depending on type of selection, this may increase or decrease genetic variation. If neutral markers are linked to selectable markers (linkage), they also may respond to selection (Van Straalen and Timmermans, 2002).

Multi-generation exposure of a population to a toxicant may result in a directional selection favoring those genotypes that are more tolerant to the chemical, which may lead to genetic adaptation of the population. Such genetically-based increased tolerance has

been demonstrated for several toxicants and populations. Lopes et al. (2006) indicated increased Cu tolerance for *D. longispina* populations, that are historically-stressed by acid mine drainage, in comparison with reference populations. Similar results were found by Lopes et al. (2004, 2005); Coors et al. (2009); Agra et al. (2010); Vidal and Horne (2003a,b) (see Chapman, 2008). The predictive assessment of adaptive abilities could be performed via multi-generation artificial selection experiments or through the concept of heritability, which can be measured through quantitative genetics (see further). Measures of this micro-evolutionary potential are heritabilities and genetic coefficients of variation. Heritability embraces two components: (1) the amount of genetic variability and (2) the potential to transmit the differences in sustaining this variability. Although genetic variability towards toxicants is detected in numerous studies, the amount of genetic variability is not necessarily heritable due to the possibility of non-additive genetic interactions: dominance and combined epistatic effects (Falconer and Mackay, 1996). Only a few studies (Chaumot et al., 2009; Klerks and Moreau, 2001) have studied the non-additive components and additive components under toxicant stress. Studying whether populations may adapt to contamination is critical for risk assessment (Chaumot et al., 2009), as numerous studies even indicate that adaptation appears to be infrequent (Klerks, 2002; Chaumot et al., 2009), because of the weakness of additive components.

When micro-evolution due to pollution occurs, increased tolerance can be considered as a positive event. However, the acquisition of genetically inherited tolerance could have long-term ecological consequences. First, natural selection may result in a reduction of genetic diversity (Lynch and Walsh, 1998). This has been indicated in several studies (Van Straalen and Timmermans, 2002; Medina et al., 2007). This in turn may lead to

(1) a decreased tolerance to other stressors (Ward and Robinson, 2005), (2) a reduced adaptive potential towards future challenges imposed by novel stressors (Van Straalen and Timmermans, 2002), or (3) a reduced fitness when the selective pressure is removed (e.g. after remediation of a polluted site), an observation which is commonly referred to as "cost of tolerance" (Medina et al., 2007). This latter phenomenon is caused by genetic between-environment correlations or between-environment trade-offs (Medina et al., 2007) and has been shown in several studies (Shirley and Sibly, 1999; Postma et al., 1995a; Levinton et al., 2003). Also, genetic correlations among fitness-related traits or trade-offs can constrain evolution (Reznick et al., 2000). This trade-off occurs when an increase in fitness due to a change in one fitness component is counter-acted by a decrease in fitness due to a concomitant change in another fitness component (Roff and Fairbairn, 2007). As such, analysis of genetic correlations does not only provide insight as to why evolution of fitness is constrained, but it can also indicate which of several individual fitness components may evolve along with fitness (due to natural selection) and in which direction.

Daphnia magna populations are the ideal test-organisms for the study of micro-evolutionary responses. They are widely used in risk assessment and they can reproduce asexually by ameiotic parthenogenesis. Thus the genetic and environmental components of variance can easily be separated in experimental design (Falconer and Mackay, 1996) (see 2.2.). Also, natural populations of *D. magna* consist out of two components: a free-living population and a seed bank of dormant eggs (i.e. ephippia). Hence, sampling of those ephippia, which are produced by sexual reproduction, is a representation of the complete gene pool. Additionally *Daphnia* (i) are amongst the freshwater species which are most sensitive to chemicals (Wogram and Lies; 2001), including Cd (ECB, 2007; Von der Ohe and Lies

2004); (ii) have demonstrated to exhibit genetic variability of Cd tolerance within populations (Baird et al., 1990; Barata et al. 1998; Barata et al., 2000b; Barata et al., 2002a,b,c), (iii) have been demonstrated to rapidly adapt to stress (Cousyn et al., 2001; Ward and Robinson, 2005; Van Doorslaer et al., 2007; Brausch and Smith, 2009) and (iv) provide ideal model species to study both genetic variation and micro-evolutionary responses in populations (Colbourne et al. 2005; Van Doorslaer et al. 2007).

2. Measurement of a micro-evolutionary potential

2.1. Experimental micro-evolution

There are three types of experimental evolutionary experiments (Bennett, 2003). The first is artificial truncation selection: only individuals that possess a desired trait are permitted to breed and found the next generation. This type of selection is well documented in animal and plant breeding studies. The second type is laboratory culling selection: where populations are exposed to a lethal environment in each generation and hence, only survivors can be found in the next generation. The third type of selection is laboratory natural selection. This type of selection, in contrast with laboratory culling selection, proceeds by soft selection rather than hard selection (see Bennett et al., 2003). Experimental micro-evolution has been applied on *Daphnia magna* populations exposed to parasites (Capaul and Ebert, 2003; Zbinden et al., 2008) and temperature stress (Van Doorslaer et al., 2010, Van Doorslaer et al., 2007). Only a few studies have studied the effects of toxicants in an experimental micro-evolution set-up (Xie and Klerks, 2003; Jansen et al., 2010; Ward and Robinson, 2005).

Jansen et al. (2010) exposed in total 125 *D. magna* clones from each of in total 8 populations to 3 pulses of 32 µg/L carbaryl during a period of 6 weeks. A replicate of each population was exposed to control conditions (in total 16 populations). In a following predation experiment, a total of Daphnids of five isolates (15 randomly selected individuals of each of the five clones from the respective population) per population were used. Their results indicate no effect of carbaryl exposure during a selection experiment on vulnerability of Daphnia to fish predation. In the experiment of Xie and Klerks (2003) field collected least killifish (*Heterandria Formosa*) were exposed to 6 mg Cd/L. The survivors of this first generation were randomly distributed over three selection lines followed for several generations. Each selection line was paired with a control line (from unexposed fish from the base population). Copper resistance was investigated in the second, third and 6th generation. Heat resistance was quantified in the second, third and 5th generation. Response to selection for Cd resistance was found rapid in the least killifish. After two generations of selection, fish from all the selection lines had a longer survival time when exposed to Cd (compared to the control lines). Moreover the Cd resistance was accompanied by cross-resistance to Cu, but a decreased resistance to higher water temperature (38°C). Ward and Robinson (2005) exposed a population of 8 *D. magna* clones to 61 µg Cd/L. Survivors from each of the Cd exposed population were randomly selected and used in a following generation of Cd and control exposure. This procedure was repeated in the following generations. An increase in Cd resistance was found within a few generations. The Cd-adapted daphnids and the control daphnids were equally sensitive to Cu and malathion, but the Cd-adapted daphids were more sensitive to phenol than the control daphnids.

2.2. Quantitative genetics

The goal of quantitative genetics is to understand how genes and environment combine to determine phenotypic variation in populations (Schwaegerle et al., 2000). Population genetics define genetic variation as "differences among individuals in a population that are due to differences in genotype" (Van straalen and Timmermans, 2002).

The minimum requirements for an evolutionary change (e.g. ability to cope with anthropogenic and natural stressors) in populations are the occurrence of natural selection and the presence of heritable variation in the selected trait (Lynch and Walsch, 1998 in Hoffmann and Merilä, 1999). Quantitative genetic variation within populations often varies in different environments (Swindell and Bouzat, 2006). These changes in quantitative genetic variation are expected to influence the ability of populations to undergo adaptive evolutionary change (Swindell and Bouzat, 2006) and are therefore of great importance to the study of evolution. For example, if the environment strongly impacts the expression of quantitative genetic variation, environmental conditions may alter the rate at which populations respond to natural and artificial selection (Swindell and Bouzat, 2006) which could influence the ability of populations to avoid extinction.

Measures of quantitative genetic variation

The phenotype (P_i) of an individual consists out a genetic component (G_i) and an environmental component (E_i):

$$P_i=G_i+E_i \quad \text{(Eq. 1.1)}$$

The genetic component consists out (1) an additive effect (= a component of the sum of individual effects of all alleles across all contributing loci), (2) a dominance effect (= a component due to non-additive effects of the two alleles at each locus, summed across all loci) and (3) an epistatic effect (= a summation consisting of effects due to specific combinations of alleles across loci that cannot be attributed to additive or dominance effects) (Eq. 1.2). The environmental component emerges due to random noise that each individual experiences to some degree.

$$G_i = A_i + D_i + Ep_i \quad \text{(Eq. 1.2)}$$

where G_i= genetic component of individual i, A_i= additive component of individual i, D_i= dominance component of individual i, Ep_i= epistatic component of individual i.

At population level, the variance at the phenotypic level can be partitioned into components. The narrow sense heritability of a trait (h^2), defined as the proportion of the phenotypic variance (V_P) accounted for by additive genetic effects V_A (i.e.V_A/V_P), is an indicator of the extent to which a trait can evolve. Heritability is one of the most fundamental concepts in quantitative genetics because it is directly related to the response to selection:

$$R = h^2 * S \quad \text{(Eq. 1.3)}$$

Where R is the response to selection (the change in the mean value of the character after selection), h^2 is heritability and S is the selection differential (the difference between the mean of the selected group, compared to the mean of the original population). This equation indicates that the larger the heritability of a character, the quicker the population changes under selection (Van Straalen and Timmermans, 2002).

Fundamental theory of natural selection

Quantitative genetic variability determines the potential for an adaptive evolutionary response and is therefore of great importance to the study of evolution. The fundamental theory of natural selection describes how natural selection operates upon the phenotype of fitness when fitness is heritable but genetically an unmeasured trait. Fitness is then defined as a measure of average reproductive success of a phenotypic class of individuals. The mean phenotype is then (Templeton, 2006):

$$\mu = \int_x x\, f(x)\, dx \quad \text{(Eq. 1.4)}$$

where x = the phenotypic value of some trait for an individual in a population

f(x) is the probability distribution that describes the frequencies of x in that population

The mean or average fitness of the population is (Templeton, 2006):

$$\bar{\omega} = \int_x \omega(x)\, f(x)\, dx \quad \text{(Eq.1.5)}$$

with $\omega(x)$ = the fitness value of those individuals sharing a common phenotypic value x

Selection can alter the mean phenotype of the population of individuals. The frequency of selected individuals is proportional to $\omega(x)\, f(x)$. So the mean phenotype of the selected individuals is (Templeton, 2006) :

$$\mu_s = \int_x \omega(x)\, f(x)\, dx \;/\; \bar{\omega} \quad \text{(Eq.1.6)}$$

Fitness is a phenotype, so what happens with the phenotype of interest is fitness itself (Templeton, 2006):

$$\mu_s = \left(\int_\omega \omega\ \omega\ f(\omega)\, d\omega\right) / \bar{\omega} = (\sigma^2 + \bar{\omega}^2) / \bar{\omega} \quad \text{(Eq.1.7)}$$

The total phenotypic variance of a trait has a genetic (V_G) and an environmental component (V_E).

The evolutionary potential of a phenotype can be determined by broad sense heritability (for asexual reproducing organisms). Broad sense heritability is defined as the genetic variance of a character, relative to the total variance. This heritability is directly related to the response to selection, and is expressed as:

$R = H^2 * S$ (Eq. 1.8.)

where R = the response to selection (= the change in the mean value of the character after selection)

$R = \mu_0 - \mu$ (Eq. 1.9)

R can be rewritten as, where $x=\omega$:

$R = V_G / \overline{X}$ (Eq. 1.10)

S = the selection differential (the difference between the mean of the selected group and the mean of the original population):

$S = \mu_s - \mu$ (Eq.1.11)

S can be rewritten as:

$S = \sigma^2 / \overline{\omega}$ (Eq.1.12)

$\Delta \overline{X} = \sigma^2_G / \overline{X}$ (Eq.1.13)

Equation 1.13 is known as Fisher's fundamental theorem of natural selection (Fisher, 1930; Houle, 1992; Templeton, 2006), which states that the increase in fitness at any time is equal to the genetic variance of fitness at that time. Dividing both sides by mean phenotype yields an expression for the proportional change of a trait. This can be rewritten as:

$\Delta \overline{X} / \overline{X} = V_G / \overline{X}^2$ (Eq.1.14)

Equation 1.14 is the relative evolvability of fitness and is related to the genetic coefficient of variation.

$V_G / \overline{X}^2 = (CV_G/100)$ (Eq. 1.15)

$CV_G = 100 * \sqrt{V_G} / \overline{X}$ (Eq. 1.16)

Heritability (H^2 or h^2) estimates may not be the most relevant predictor of selection response. A heritability estimate provides a prediction of the absolute response to selection via $R^2 = h^2 * S$ (see above). However, it may also be important to know the relative change of a trait. The use of additive genetic coefficient of variation (CV_A) or genetic coefficient of variation (CV_G) would provide a prediction of the proportional response to selection (Houle, 1992 and Klerks et al., 2011). Use of CV_A has the added benefit that the variable itself is not affected by the environmental variance (Klerks et al., 2011).

3. Introduction to the model organism *Daphnia magna* (Flößner,2000)

3.1. Systematic classification and morphology

Daphnia magna is divided into the order of the cladocerans (waterfleas), which is divided into 11 families, 80 genera and about 400 species. According to the World register of Marine species (WoRMS,2010) the current taxonomy is as follows:

Kingdom	Animalia
Phylum	Arthropoda
Subphylum	Crustacea
Class	Branchiopoda
Subclass	Phyllopoda
Order	Diplostraca
Suborder	Cladocera
Infraorder	Anomopoda
Family	Daphniidae
Genus	*Daphnia* O.F. Müller, 1785
Species	*Daphnia magna* Straus, 1820

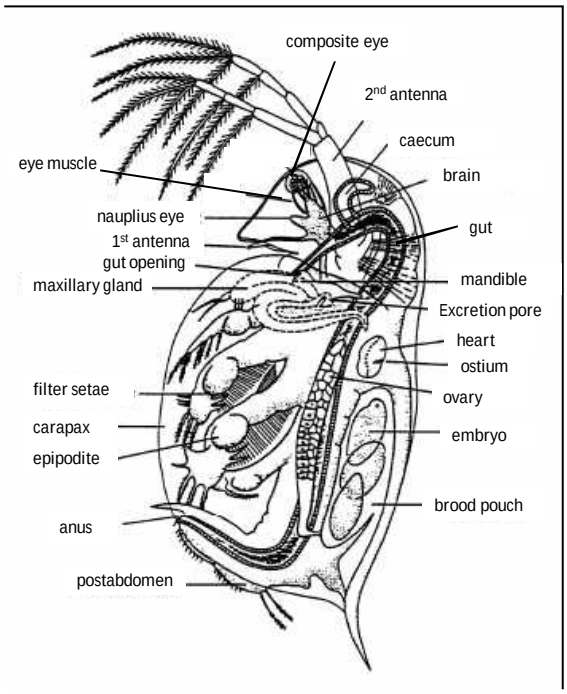

Figure 1.2: Anatomy of a female Daphnia magna, after Barnes (1980)

D. magna is one of the largest daphnids with adult females growing up to 6 mm. Males are smaller, with a flattened frontal portion of the head capsule, elongated first antennae and lacking the abdominal process which forms the boundary of the brood chamber in females (Olmstead and LeBlanc, 2000). In female daphnids, oviducts open in a dorsal brood pouch or brood chamber, which lies inside the carapax. Eggs develop to

juveniles inside this brood chamber and juveniles can be released by a ventral flexion of the postabdomen. When the young leave the brood chamber, the skeleton is moulted and a new batch of eggs is released into the new brood chamber. *D. magna* are usually pale and transparent, facilitating microscopic observations (Goldmann *et al.*, 1999).

3.2. Ecotoxicological and physiological aspects

Daphnia magna has a Holarctic distribution and lives in small to medium-sized shallow freshwater pools and ponds with moderate to low fish predation (De Gelas and De Meester, 2005). *Daphnia magna* tolerates brackish water up to 8% and pH values ranging from 5.6 to 10.7. The genus *Daphnia,* representing a large part of the freshwater zooplankton community, forms an indispensible element in the freshwater food web. It is one of the most important consumers of primary producers, while in turn it is an important food source for both invertebrate and vertebrate predators. Presence or absence of *Daphnia* can have considerable implications for the ecological quality of an aquatic system (Hebert, 1978).

Reproduction in *D. magna* occurs through cyclical parthenogenesis (Zaffagnini, 1987). Under favourable conditions females produce diploid parthenogenetic eggs in the ovary. Oogenesis is in this case not fully meiotic nor strictly mitotic. The eggs are deposited into the brood pouch, in which they develop to clones that are genetically identical to the adult. These juveniles are sexually mature after four to five molting stadia, which takes about seven days at a temperature of 20°C. Certain environmental triggers (e.g. food limitation, high population densities, a decreasing photoperiod, desiccation of the habitat) can induce a sexual reproduction phase. In that case, male organisms appear, which are also produced parthenogenetically and consequently are genetically identical to the females that produced

them. Subsequently, females deposit haploid eggs (two per brood), which need to be fertilized by the males to ensure further development to resting eggs. These resting eggs are encapsulated by multiple layers of membranes, formed by a transformation of the brood pouch. The result is a saddle-like structure called the ephippium, containing fertilized resting eggs. This ephippium protects the eggs against adverse environmental conditions. When the adverse environmental conditions are over, the ephippia develop to parthenogenetically reproducing females and a new parthenogenetic phase can begin. In the laboratory, favorable environmental conditions can be maintained continuously, allowing for an elimination of the sexual phase and the maintenance of genetically identical female clones.

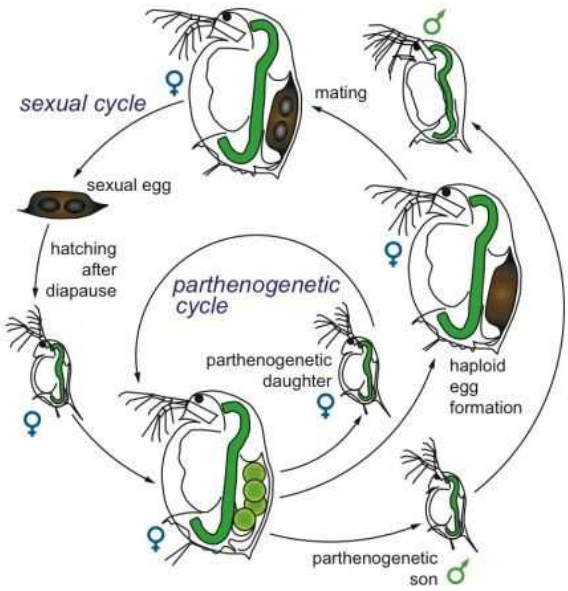

Figure 1.3: Reproduction system of *Daphnia magna*.

The hatching of resting eggs is introduced by osmotic water uptake. Resting eggs develop into females. Juveniles of the Daphniidae undergo up to 7 moults. Daphnia magna moult every 2 to 3 days. The moult itself takes only a few minutes. Organisms are able to grow in between two moults when the newly synthesized carapax is not hardened yet. The

lifespan is dependent on temperature and food supply; the average lifespan at 8°C is 108 days, while at 28 °C this is only 29 days. Males always have shorter lifespan than females.

4. Scope of the research

This PhD study was aimed at addressing potential micro-evolutionary effects of chemicals on natural *Daphnia magna* populations that have until now, insufficiently been addressed in conventional ecotoxicology and risk assessment. Cadmium was used throughout the research as a model chemical.

In contrast with laboratory populations, field populations may be exposed to long-term chemical stress and do exhibit genetic variation towards stress. The genetic variability within a population determines the micro-evolutionary potential of a population exposed to stress. Natural selection may act upon this genetic variability and this may result in an increase of the mean fitness of the population. Increased tolerance to pollution can be considered as an ecologically positive event. However, this may have adverse long-term ecological consequences: i.e. a reduction in genetic diversity (Lynch and Walsh, 1998), which may lead to (1) a decreased tolerance to other stressors (Ward and Robinson, 2005), (2) a reduced adaptive potential towards future challenges imposed by novel stressors (Van Straalen and Timmermans, 2002) or (3) a reduced fitness when the selective pressure is removed (e.g. after remediation of a polluted site) an observation which is commonly referred to as "cost of tolerance" (Medina et al., 2007). Irrespective of wheter micro-evolutionary responses under chemical stress are considered "positive" (increased tolerance) or "negative" (cost of adaptation), it is of interest to know how the micro-evolutionary potential is affected on a function of chemical concentration. In chapter 2, we investigated the micro-evolutionary potential (expressed as CV_G and H^2) of a natural *Daphnia*

magna population under increasing Cd stress (from 1-22 µg Cd/L) compared to the control. We also determined if there was a cost of tolerance under this Cd range.

In conventional ecotoxicology, tests are usually conducted under standardized conditions. For instance, temperature is often controlled at 20°C in *Daphnia magna* test. However, it is well-known that an increased temperature (i.e. in context of global warming) often results in a higher toxicity of chemicals (Heugens et al., 2006). The effects of temperature on micro-evolutionary responses (micro-evolutionary potential, cost of adaptation) to chemical exposure have, however, not been studied. Besides cost of adaptation, evolution can also be constrained by genetic correlations among fitness-related traits (Reznick et al., 2000), also commonly referred to as trade-offs. Analysis of such genetic correlations does not only provide insight as to why evolution of fitness is constrained, but it can also indicate which of several individual fitness components may evolve along with fitness (due to natural selection) and in which direction. Therefore, in chapter 3, we tested a set of hypotheses related to (i) the micro-evolutionary potential, constraints and possible consequences in a natural population of a *Daphnia magna* exposed to a single sublethal cadmium concentration (5 µg Cd/L compared to a control) and (ii) the influence of temperature thereupon.

While in chapter 2 and 3, we studied the total amount of genetic variability, it is recognized that what is genetically determined is not necessarily heritable across sexual generations because of non-additive components of genetic variability. Previous studies indeed indicate that adaptation to chemicals in the field is generally infrequent (Klerks, 2002; Chaumot et al., 2009), possibly because of a small additive genetic variance. Chaumot et al. (2009) stated (based on weakness of additive components for Cd tolerance) that

exceptional cases of adaptation of field populations would be permitted only by the fixation of rare alleles (Woods and Hoffmann, 2000). Therefore, we examined the additive and non-additive components of a natural *D. magna* population under Cd stress at two temperatures (20°C and 24°C) in chapter 4. In total we crossed 20 parental clones.

The previous chapters are focused on the micro-evolutionary potential and constraints (between-trait correlations and cost of tolerance) of one *Daphnia magna* population, although variability between populations is possible. In chapter 5, we therefore studied the effect of a sublethal Cd-concentration on 11 *D. magna* populations in terms of micro-evolutionary potential (i.e. within population variability).

Besides, quantitative genetics (chapter 2 - chapter 5), the predictive assessment of micro-evolutionary responses could be performed via multi-generational artificial selection experiments. In chapter 6, we performed this type of experiment, with one *Daphnia magna* population exposed to a Cd concentration range between 0 and 22 µg Cd/L during a 203 day micro-evolution experiment under semi-field conditions. Afterwards, we determined if the long-term Cd exposed populations (range between 2.2 – 22 µg Cd/L) had a higher fitness (positive event) exposed to Cd compared to the long-term control exposed population and the start population (= population kept under laboratory circumstances) and if there was a cost of adaptation (negative event).

Finally, we compared our results on effects of Cd on micro-evolutionary potential with the conventionally derived PNEC and EQS in risk assessment, but also with NOEC of *D. magna* clones used in EU Cd risk assessment (chapter 7). In addition, standardized 21-day tests were performed using 7 European monoclonal *Daphnia magna* populations to

determine effect concentrations (NOECs, 21d-EC_{10}s and 21-day EC_{50}s) tested under same lab conditions and at the same time.

Measure of micro-evolutionary change	Test organism	Chapter	Stressor	Research-questions	Measured endpoints
Quantitative genetics	1 *Daphnia magna* population	2	Cd concentration range: 0-22 µg Cd/L	Cd effect on population mean? Cd effect on micro-evolutionary potential? Is there a cost of tolerance?	Population mean CV_G and H^2 Between environment (Control and Cd) genetic correlations
		3	Control and 5 µg Cd/L under two temperature treatments (20°C and 24°C)	Is there a temperature effect on micro-evolutionary aspects? - Increased temperature result in a larger Cd effect? - Increased stress results in an increased micro-evolutionary potential under Cd stress? - Is there a cost of tolerance? - Among fitness-traits genetic correlations?	Population mean CV_G and H^2 Between environment (Control and Cd) genetic correlations Between trait-genetic correlations
		4	Control and 5 µg Cd/L under two temperature treatments: (20°C and 24°C)	Is there a temperature and Cd effect on: - Additive components - Dominance components - h^2	$CV_A/h^2/\sigma^2_A$ σ^2_D
	11 *Daphnia magna* populations	5	Control and 5 µg Cd/L	Cd effect on micro-evolutionary potential of 11 *Daphnia magna* populations?	CV_G and H^2
Micro-evolutionary experiment	1 *Daphnia magna* population	6	Long-term Cd exposure between 0-22 µg Cd/L	At which Cd concentration is there a rapid micro-evolutionary response? Is there a cost of tolerance?	Population mean

Chapter 7

1. Are there micro-evolutionary effects at conventionally derived PNEC and EQS?

2. Are there micro-evolutionary effects at conventionally derived NOEC's (use of monoclonal laboratory *Daphnia magna* populations)

Chapter 2: The micro-evolutionary potential of a natural *Daphnia magna* population exposed to Cd stress

Abstract- Conventional risk assessment does not account for potential micro-evolutionary responses of natural populations to chemical stress. In the present study, we determined the genetic coefficient of variation (CV_G) and broad sense heritability (H^2) as measures of genetic variability of total reproduction (R_0) and population growth rate (r_m) by means of 21-day life-table experiments with 11 genetically distinct clones from a natural *Daphnia magna* population exposed to a control and Cd concentrations between 0.89 and 18.9 µg Cd/L. We also determined a cost of tolerance (i.e. negative genetic correlations between environments) within this Cd range. Based on significantly higher genetic variation of fitness in a Cd treatment vs. the control, a higher micro-evolutionary potential was observed at 1.9 µg Cd/L (based only on $CV_G(r_m)$) and at 18.9 µg Cd/L (based on $CV_G(R_0)$, $CV_G(r_m)$, and $H^2(r_m)$). No negative correlations between control and Cd treatments were found, suggesting no cost of Cd tolerance in higher Cd environments.

1. Introduction

Conventional risk assessment of chemicals is based on the analysis of the mean population response of selected life-history traits and does not take into account the genetic variability of that response (Forbes and Forbes, 1994; Barata et al., 2000b). Indeed, genetic variability is often minimized in ecotoxicology by using monoclonal laboratory test populations, such as in the case of *Daphnia* sp. toxicity tests (Baird and Barata, 1998). Although this approach facilitates standardization and guarantees low variability and high reproducibility of test results, it is unreliable to predict long-term (multi-generation) effects of chemical exposure on natural populations.

Indeed, natural populations are generally characterized by genetic variability upon which natural selection may act. Thus, multi-generational exposure of a population to a chemical may result in a directional selection favoring those genotypes that are more tolerant to the chemical, which may lead to genetic adaption of the population. For instance, genetically-based increased tolerance to Cu has been demonstrated for *D. longispina* populations that are historically-stressed by acid mine drainage, in comparison with reference populations (Lopes et al., 2006). Similarly, Coors et al. (2009) reported local genetic adaptation, expressed as an increased tolerance to the insecticide carbaryl, in *Daphnia magna* populations from ponds that are impacted by increased agricultural land use intensity.

The evolution of increased tolerance to pollution may be important for ecological risk assessment, because it may allow the persistence of populations in contaminated habitats. This could be considered as an ecologically positive event. On the other hand, increased tolerance of the population to a chemical stress due to directional selection of tolerant

genotypes may also have adverse long-term ecological consequences. Indeed, natural selection results by definition in a reduction of the genetic diversity (Lynch and Walsh, 1998). This in turn may lead to a decreased tolerance to other stressors (Ward and Robinson, 2005), a reduced adaptive potential towards future challenges imposed by novel stressors (Van Straalen and Timmermans, 2002) or a reduced fitness when the selective pressure is removed (e.g. after remediation of a polluted site) an observation which is commonly referred to as "cost of tolerance" (Medina et al., 2007). This phenomenon is caused by genetic between-environment correlations or between-environment trade-offs (Medina et al., 2007). For example, Shirley and Sibly (1999) observed that a *Drosophila* population cultured under high Cd stress during several generations exhibited lower reproduction when reared in clean media afterwards. Similarly Postma et al. (1995a) showed that Cd tolerant *Chironomus riparius* populations had lower fitness when reared in a clean environment. Levinton et al. (2003) indicated that after clean-up of a Cd polluted site, the loss of tolerance in *L. Hoffmeisteri* had a genetic basis. Irrespective of wheter micro-evolutionary responses under chemical stress are considered "positive" or "negative", it is of interest to know how the micro-evolutionary potential is affected on a function of chemical concentration.

Although knowledge of between environment trade-offs are considered a key element for incorporating micro-evolution in the environmental risk assessment paradigm (Medina et al., 2007), this type of limited information is only available for highly contaminated environments (see reviews in Medina et al., 2007 and Morgan et al., 2007). Knowledge of such trade-offs in contaminated systems with a range of Cd concentrations is completely lacking.

The aims of the present study were therefore to test a hypotheses related to the micro-evolutionary potential of a natural population of *Daphnia magna* exposed to a cadmium concentration range. The first hypothesis - as proposed by Barata et al. (2000b) - is that a population exposed to cadmium will exhibit more genetic variation for life-history traits than a control population. As with increasing Cd concentrations, there will be a lower fitness, and following Hoffmann and Hercus (2000), this will in turn result in a higher genetic variability of life-history traits in a high cadmium environment (hypothesis 1). We hypothesize that there may be a cost of tolerance which will be reflected by a negative genetic correlation of fitness between control and Cd treatments (hypothesis 2).

The micro-evolutionary potential of a population exposed to a chemical stress can be based on the measurement of genetic variability. This can be understood as follows. The minimum requirements for a micro-evolutionary change of any phenotypic trait in a population are (i) the presence of genetically heritable variation of that trait, (ii) the occurrence of natural selection and (iii) a genetic correlation of that trait with fitness (Lynch and Walsch, 1998; Templeton, 2006; Hoffmann and Hercus, 2000; Chaumot et al., 2009). For clonally reproducing organisms, like *Daphnia magna*, there are different ways to standardize the level of genetic variation of a (fitness) trait (e.g. total reproduction, R_0 or intrinsic rate of increase, r_m) for comparative purposes (Barata et al., 2002b). First, the total genetic variance (V_G) can be presented as a proportion of the total phenotypic variance (V_P), and this ratio is called the (broad sense) heritability (H^2) (Lynch and Walsh, 1998):

$$H^2 = V_G / V_P \quad \text{(Eq. 2.1)}$$

Second, Houle (1992) proposed the genetic coefficient of variation (CV_G) to be a possibly better measure of the ability of fitness traits to evolve, compared to H^2:

CV_G (%) = 100 * √V_G / $\overline{X}$ (Eq. 2.2)

With CV_G = the genetic coefficient of variation, V_G = the total genetic variance, and $\overline{X}$ = the population mean of the (fitness) trait. Since there is currently no consensus about which of these measures is the better measure of micro-evolutionary potential under stress, we decided to consider both in the present study.

We performed a 21-day life table experiment with 11 genetically distinct clonal lineages established from ephippial eggs from a single natural *D. magna* population under a control treatment and under Cd treatments of 0.89 to 18.9 µg/L (measured dissolved concentrations). This allowed us to determine CV_G and H^2 as the measurements of genetic variability of two fitness traits, i.e. R_0 and r_m. Those fitness traits are commonly used in ecotoxicology with *Daphnia magna* population. By measuring the various life-history traits of individuals of each genotype kept under different environmental conditions, estimates of quantitative genetic variation were obtained as measures of the micro-evolutionary potential of the population (Barata et al., 2002b; Lynch and Walsh, 1998).

2. Materials and methods

2.1. General culture and exposure conditions

The maintenance and exposures of all clones of the natural and the laboratory populations were performed at 20°C and under a light:dark cycle of 16h:8h. Daphnids were fed daily with a 3:1 mixture (based on cell numbers) of the algae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii.* Culture maintenance and exposures were performed in modified M4-medium. This medium is different from the original composition

of M4 medium (Elendt and Bias, 1990) as follows: Na_2EDTA and $FeSO_4$ were omitted and replaced with natural dissolved organic matter (DOM) at a concentration of 4 mg dissolved organic carbon (DOC)/L. The DOM was collected from a small creek (Ruisseau de St. Martin, Bihain, Belgium) using a portable reverse osmosis system (PROS/2) (Sun et al., 1995). This modified M4 medium has a hardness of 250 mg $CaCO_3$/L and pH of 7.6. Exposure media were prepared and spiked with Cd 24h to 48h prior to use and subsequently stored at 20°C in 25 or 50 L polyethylene vessels until use.

2.2. Establishment and maintenance of clonal lineages of the natural population cultures

Sediment containing *Daphnia* ephippia was collected from the Kasteelvijver pond in the nature reserve Blankaart (Diksmuide, Belgium) using a Van Veen grab and a sediment corer in October 2007 (Figure 2.1). The samples were transferred to the laboratory and ephippia were isolated. Ephippia of *D. magna* were identified (Vandekerkhove et al., 2004) and subsequently hatched at 20°C under continuous light (4000 lux) in modified M4 medium (see 2.1.). Each ephippium was hatched individually in a 50 mL polyethylene vessel and a single hatchling from each ephippium was selected to establish a clonal lineage. Ephippial eggs of *D. magna* are produced by sexual reproduction (Ebert et al., 1993) so each clonal lineage can be considered genetically distinct (Barata et al., 2000b). Next, the juvenile hatchlings were assigned a clone name and were placed individually in a 50 mL polyethylene cup filled with modified M4 medium and kept at 20°C and under a light:dark cycle of 16h:8h. The organisms were fed daily with a 3:1 mixture (based on cell numbers) of the algae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* equivalent to 250 µg dry wt/*Daphnia*, 500 µg dry wt/*Daphnia* and 750 µg dry wt/*Daphnia* in the first, second and third week of their life, respectively. Juvenile offspring of the third brood of the hatchlings

were transferred to 200 mL polyethylene vessels and the next generations of each clone were fed 1.7 mg dry wt of algae per day per 200 mL. The culture medium was refreshed once a week. With every medium renewal, the next generation of each clone was established by randomly picking 2 to 4 juveniles and 1 or 2 adult daphnids (daphnids carrying eggs in the brood pouch) of the previous generation. Thus, with every renewal, 3 to 6 daphnids were placed in 200 mL polyethylene vessels. After more than two years of culturing following this procedure, a total of 11 randomly selected clonal lineages were used for the life-table experiments.

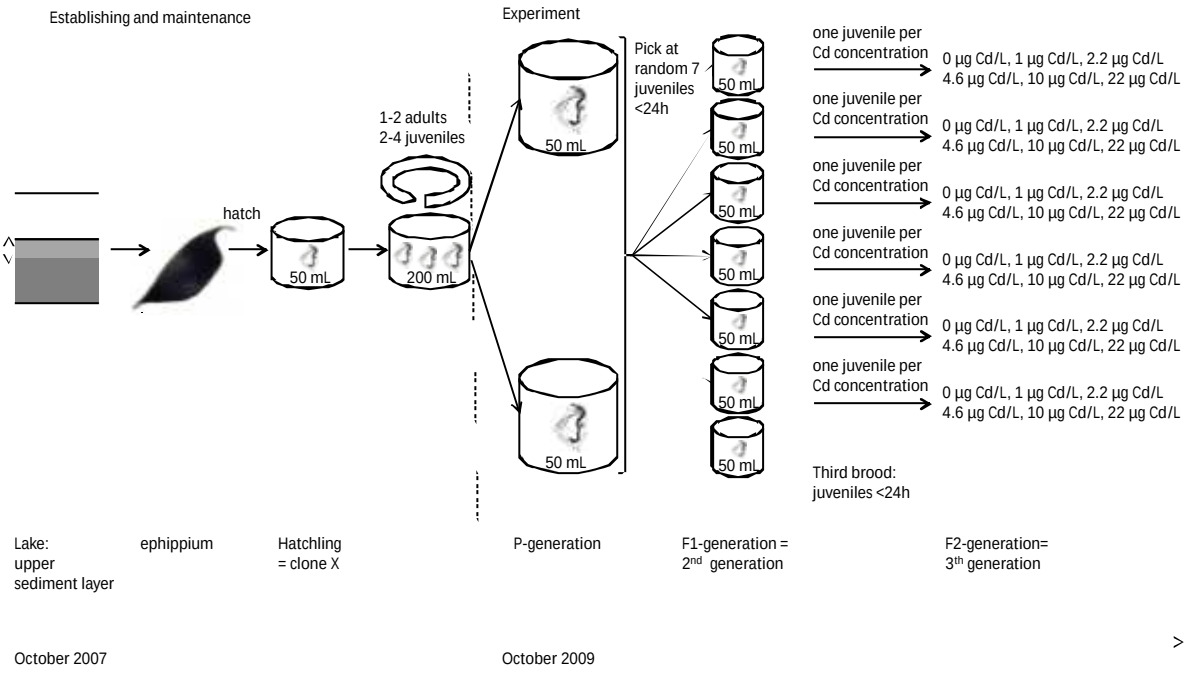

Figure 2.1: Schematic overview of the experimental design that was followed for each clonal lineage originating from the field population.

2.3. Test design of the exposures to control and Cd

The test design is scheduled in Figure 2.1. In a first step, two adult individuals from each of the 11 field clones were transferred individually to separate 50 mL polyethylene beakers (= P generation in Figure 2.1). For each clone, juveniles (<24h) produced by these

two adults were pooled together and 7 juveniles (<24h) were randomly picked out, to start the second generation (2^{nd} generation, i.e. F1, in Figure 2.1). Each juvenile was transferred individually to a separate 50 mL polyethylene beaker. The individuals in this second generation (F1) then served as the mothers for producing the following generation. At the third brood, six juveniles (<24h) (F2) from six different mother organisms (F1) were selected and were placed individually in 50 mL polyethylene vessels with modified M4 medium and with a Cd range between 1 and 22 µg Cd/L (added as $CdCl_2 \cdot H_2O$) including a control (no added Cd). As such, maternal effects can be ruled out in the estimation of genetic variance, as for each clone in each Cd concentration, each of the six replicate individuals (juveniles) being exposed originated from a different mother organism. Thus, during statistical estimation of the two variance components (i.e. genetic and residual, see 2.5) maternal variance is 100% included in the residual variance (Lynch and Walsh, 1998). All Cd exposures with all field clones were simultaneously initiated, allowing a comparison that is not biased by temporal variability of the cultures. Medium renewal was three times a week and organisms were fed daily with 250 µg dry wt/individual, 500 µg dry wt/individual and 750 µg dry wt/individual in the first, second and third week of their life, respectively. Based on daily observations the following traits were determined: population growth rate (r_m) survival and total reproduction at day 21 (R_0) (=total reproduction). Population growth rate (r_m) was calculated according to Euler-Lotka equation (Lotka, 1913):

$$1 = \sum_{x=0}^{x=20} l_x m_x e^{-rmx} \qquad \text{(Eq. 2.3)}$$

Where l_x is the fraction of surviving females until age x, m_x is the number of offspring produced by a surviving female between age x and x+1. The r_m was calculated separately for

each individual. pH was measured at every renewal of the old medium per Cd concentration and in the beginning of the test of the new medium. Old medium is defined as the medium in test vessels just prior to the renewal of the medium, whereas new medium is the medium in the vessels, just after renewal and before addition of algae.

2.4. Chemical analyses

During the experiments samples for dissolved Cd analysis were taken once a week of the old and new medium. Every week, 10 mL samples of the old and new medium were filtered through a 0.45 µm filter (Acrodisc Filter, Supor Membrane, PALL, Newquay, Cornwall, U.K.), were collected in polypropylene tubes and were acidified with 0.14 mol/L HNO_3 (Normaton Ultrapure 69% HNO_3, Prolabo) prior to storage. Samples for Cd analysis were stored at 4°C in the dark until analysis. Cadmium concentrations were measured using ICP-MS (inductive coupled plasma mass spectrometry, Perkin-Elmer Elan DRC-e, Wellesley, MA,USA). Dissolved Organic Carbon (DOC) samples were taken at the beginning of the experiment of the new medium and at the end of the experiment (day 21) of the old medium. Samples for DOC analysis were filtered through a 0.45 µm filter (Acrodisc Filter, Supor Membrane, PALL, Newquay, Cornwall, U.K.) and DOC was measured with a TOC analyzer (TOC5000, Shimadzu, Duisburg, Germany) as non purgeable organic carbon (NPOC). This analysis involves the removal of inorganic carbon by acidification and subsequent purging of CO_2 with N_2 gas prior to analysis.

2.5. Statistical analyses

Genetic variation of total reproduction (R_0) and population growth rate (r_m) was compared among the different Cd treatments using the genotypic coefficient of variation

(CV_G, Eq. 2.2) and broad sense heritability (H^2, Eq. 2.1) rather than the genetic variance (V_G) itself. For each Cd treatment, V_G and the environmental (or residual) variance (V_E) were estimated using the method of the moments with appropriate accounting for unequal sample sizes among clones (Searle et al., 1992; Lynch and Walsh, 1998) (See supplementary Material S2.1).

Construction of confidence intervals and hypothesis testing was performed using nonparametric random bootstrap resampling (5000 samples) with replacement of clones (Lynch and Walsh, 1998; Messiaen et al., 2010) (See Supplementary Material S2.1). If in a run the V_G turned out to be negative, it was set to zero for further calculations (Lynch and Walsh, 1998). The median values (50th percentile) and the 2.5th and 97.5th percentile of CV_G, H^2 and $\overline{X}$ (population mean) are reported. If more than 95% of the calculations yielded CV_G(Cd) > CV_G(control) or H^2(Cd) > H^2(control), the CV_G or H^2 in the Cd treatment was considered significantly higher than in the control. The population mean in a Cd treatment was considered significantly lower than in the control if more than 95% of the calculations yielded $\overline{X}$ (control) > $\overline{X}$ (Cd) (i.e. equivalent to a one-sided test at the 0.05 significance level). All calculations were performed in MATLAB 7.5.0.342 (Mathworks Inc) software.

Finally, genetic correlations among environments (Cd treatment vs. control treatment) were calculated for r_m and R_0 (to test for a fitness trade-off between the Cd-contaminated and the non-exposed environment) (Lynch et al., 1998):

$$\rho_{G,control,Cd} = cov(trait_{control}, trait_{Cd}) / \sqrt{(V_{G\ trait,control} * V_{G,\ trait,\ Cd})} \quad \text{(Eq. 2.4)}$$

Where the genetic variances of the traits are calculated as above and where the covariance across environments is estimated from the covariance of clone means (Via, 1984; Lynch et al., 1998).

Confidence interval construction and hypothesis testing for genetic correlations were conducted as outlined above. If a bootstrap run resulted in either of the two variances being equal or below zero, the genetic correlation was set to zero. We considered a genetic correlation (between traits or between environments) significantly different from zero if ρ_G >0 in >95% of the calculations (positive correlation) or if ρ_G <0 in >95% of the calculations (negative correlation).

3. Results

3.1. Physico-chemistry of test media

The physico-chemistry of the test media is presented as supportive information (Table S2.1). DOC ranged between 4.6 and 6.0 mg/L and pH between 7.6 and 7.8. The mean dissolved Cd concentrations (mean of old and new medium) differed at most 17% from the nominal Cd concentration. The Cd concentration in the old medium was on average 21% lower than in the new medium.

3.2. Population means

Values of total reproduction (R_0) and population growth rate (r_m) for all individuals, all clones and Cd treatments are given in Supplementary material (Table S2.2 – Table S2.13). Population means of all traits in the four treatments are reported in Table 2.1. The population mean of R_0 and r_m decreased monotonously with increasing Cd concentrations.

The *D. magna* population exposed to 1.92 µg Cd/L exhibited, compared to the control, a significantly lower mean reproduction during 21 days (R_0), and a lower mean population growth rate (r_m).

Table 2.1: Median of simulated population means (=mean of clone means) of total reproduction (R_0) and population growth rate (r_m). Numbers between brackets represents the 95% confidence interval. The p-values represent the fraction of bootstrap calculations that yielded lower values in the Cd treatment than in the control treatment (see Materials and Methods for details). Significant differences ($p<0.05$) are marked with an asterisk (*). Numbers between parentheses indicates the % difference between the Cd and the control treatment.

Cd concentration (µg Cd/L)	Population mean of total reproduction (R_0)	p-value (R_0)	Population mean of (r_m)	p-value (r_m)
<0.1	76.82 [65.33 - 88.94]		0.34 [0.32 – 0.36]	
0.89	68.35 [58.56 - 77.82] (-11%)	0.08	0.33 [0.30-0.36] (-3%)	0.14
1.92	63.83* [51.83 - 75.04] (-17%)	0.01	0.31* [0.27 – 0.35] (-9%)	0.03
3.96	57.79* [44.81 - 72.64] (-25%)	<0.01	0.31* [0.28 – 0.33] (-9%)	0.01
8.34	40.29* [28.99 - 54.12] (-48%)	<0.01	0.29* [0.25- 0.32] (-15%)	<0.01
18.87	6.09* [3.52 - 9.08] (-92%)	<0.01	0.13* [0.08 – 0.19] (-62%)	<0.01

3.3. Genetic coefficients of variation and broad sense heritability (H^2)

Genetic coefficients of variation (CV_G) and H^2 of the different traits are shown in Figure 2.2. For R_0, the CV_G and H^2 were significantly greater than 0 at control, at 0.9 µg Cd/L, at 4.0 µg Cd/L and at 18.9 µg Cd/L, indicating that there is significant genetic variation of this trait at those Cd concentrations. The $CV_G(R_0)$ was 22.4% in the control and exhibited a monotonically increasing trend with increasing Cd concentrations, i.e. from 17.4% at 0.9 µg Cd/L to 70.7% at 18.9 µg/L. Only at 18.9 µg Cd/L the $CV_G(R_0)$ was significantly higher compared to the control. $H^2(R_0)$ was 0.37 in the control and varied between 0.19 and 0.43

among all Cd treatments (Figure 2.2), but no significant differences between any Cd treatment and with the control were detected.

For r_m, CV_G and H^2 were greater than 0 at 4.0 µg Cd/L and at 18.9 µg Cd/L, indicating that only at those concentrations there is a significant genetic variation within this *D. magna* population (Figure 2.2). $CV_G(r_m)$ was 6.5% in the control, and remained relatively low (between 5.3% and 16.9%) up to a concentration of 8.3 µg/L. However, at 1.9 µg Cd/L and at 18.9 µg Cd/L, the $CV_G(r_m)$ was significantly higher than in the control. A slightly different pattern was observed for $H^2(r_m)$, with relatively low values between 0.03 and 0.23 for concentrations up to 8.3 µg Cd/L, none of which was significantly higher than in the control. At 18.9 µg Cd/L, the $H^2(r_m)$ of 0.49 was significantly higher than in the control.

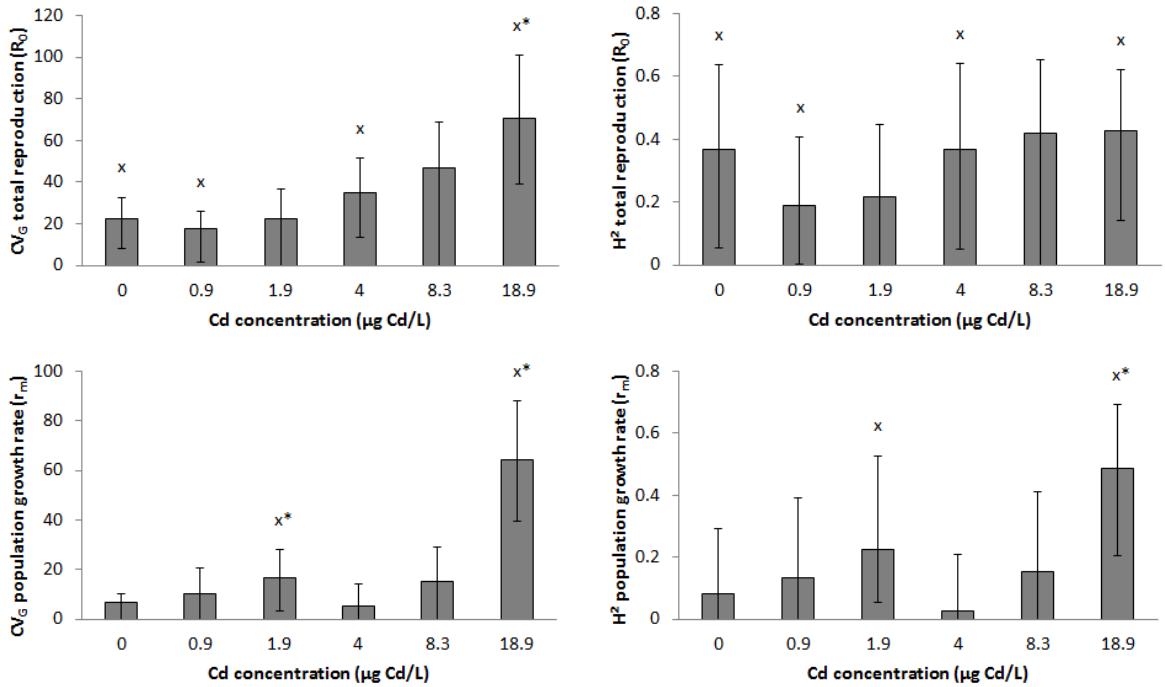

Figure 2.2: Median genetic coefficients of variation (CV_G, %) and broad sense heritabilities (H^2) for different fitness traits in a *D. magna* population. Error bars represent the 95% confidence intervals. An asterisk (*) indicates a significant difference between the Cd and the control treatment ($p<0.05$). x indicates significantly >0.

3.4. Genetic correlations between traits and between environments

For total reproduction (R_0), we found a significant positive correlation between control and 1.9 µg Cd/L, 4.0 µg Cd/L, 8.3 µg Cd/L. For r_m, no significant correlations were found (Table 2.2).

Table 2.2: Median genetic correlations between control and Cd concentration (ρControl, Cd) for total reproduction (R_0) and population growth rate (r_m). Numbers between brackets represent the 5th and 95th percentile genetic correlation coefficient between traits. An asterisk (*) indicates a significant between-environment correlation ($p<0.05$).

Cd concentration (µg Cd/L)	ρ_{R0} (Control, Cd)	ρ_{rm} (Control, Cd)
0.89	0.65 [0 - 1.35]	1.91 [0 - 4.84]
1.92	0.96 [0.35 - 1.53]*	1.49 [0 - 3.40]
3.96	0.98[0.78 - 1.66]*	1.44 [0 - 7.09]
8.34	0.74 [0.40 - 1.24]*	1.58 [0 - 4.33]
18.87	0.26 [-0.25 - 0.99]	0.08 [-0.07 – 2.20]

4. Discussion

This present study investigated the micro-evolutionary potential of a natural population of *Daphnia magna* exposed to a Cd concentration range between 0 and 18.9 µg Cd/L. Our results show that none of the two fitness traits considered here exhibited a significant genetic variation (CV_G or H^2) under every condition, which suggests that not under all conditions tested there is evolutionary potential of the population. It has to be noted that the CV_G and H^2 determined in the present study represent total genetic variation based on interclonal variation (i.e. the sum of the additive, epistatis and dominance components of genetic variation) and that the additive genetic variation could be a more precise estimate of

evolutionary potential (with total genetic variation being an upper limit of the additive variation).

The estimated values of CV_G and H^2 can also be used to test the hypothesis if the populations exhibit more genetic variation for life-history traits under Cd exposure and under control exposure. The $CV_G(R_0)$ was 22.4% in the control and exhibited a monotonically increasing trend with increasing Cd concentrations, i.e. from 17.4% at 0.89 µg Cd/L to 70.7% at 18.9 µg/L. Only at 18.9 µg Cd/L the $CV_G(R_0)$ was significantly higher compared to the control. Thus, the hypothesis proposed by Hoffmann and Parsons (1991), i.e. that increased stress (in this case Cd stress) is expected to result in increased genetic variation, is supported by the results of the present study. Yet, there is still an on-going debate on the mechanistic explanation for such observation (Hoffman and Hercus, 2000). The increase of CV_G may increase the micro-evolutionary potential under Cd stress compared to the control and may eventually lead to a stronger reduction of clonal diversity (compared to a control). $H^2(R_0)$ was 0.37 in the control and varied between 0.19 and 0.43 among all Cd treatments (Figure 2.2) but no significant differences with the control were detected. Thus, although both CV_G and H^2 are both standardized measures of genetic variation, their response to increasing Cd concentrations exhibits a different pattern. This leads to different conclusions regarding the micro-evolutionary potential of the natural *D. magna* population, i.e. a significant higher micro-evolutionary potential at 18.9 µg/L (compared to the control) with CV_G, but no such response with H^2. This finding is in line with Houle (1992), who reported, based on a meta-analysis of quantitative genetics studies, that genetic coefficients of variation and heritabilities are not necessarily correlated. Hence, he pointed out that these two standardized measures of genetic variability may have a different ecological significance.

Thus, we suggest that these two measures, when applied to the fitness traits, may also have different capacities to predict micro-evolutionary potential in the context of responses to chemical stress.

While for *Daphnia* sp., the total reproduction (R_0) is the endpoint which is most frequently used in ecotoxicity studies and in risk assessment, population growth rate (r_m) is generally considered to be a better measure of population-level responses to chemicals, because it is an integrative measure of fitness and more closely related to actual fitness in the field (Hooper et al., 2008; Forbes and Calow, 1999). The genetic variation of r_m exhibited a slightly different pattern in response to Cd than R_0. $CV_G(r_m)$ was 6.5% in the control and remained relatively low (between 5.3% and 16.9%) up to a concentration of 8.3 µg/L. However, at 1.9 µg Cd/L the $CV_G(r_m)$ of only 16.9% was significantly higher than in the control. At 18.9 µg/L, the $CV_G(r_m)$ peaked to 64.3%, representing a 10-fold (significant) increase compared to the control. Thus, based on $CV_G(r_m)$ a micro-evolutionary response is expected at 1.9 and 18.9 µg Cd/L, but not in-between or at the control. A slightly different pattern was observed for $H^2(r_m)$, with relatively low values between 0.03 and 0.23 for concentrations up to 8.3 µg Cd/L, none of which was significantly higher than in the control. At 18.9 µg Cd/L the $H^2(r_m)$ of 0.49 was significantly higher (6-fold) than in the control. Thus, based on $H^2(r_m)$, an increased expression of genetic variability is seen at 18.9 µg Cd/L. This may increase the micro-evolutionary potential under Cd stress and may lead to stronger reduction of clonal diversity in periods of asexual reproduction.

As in the present study only a single natural population was investigated, our findings should be interpreted with caution. Comparison with the study of Barata et al. (2000b) clearly illustrates this point. They observed that CV_G of total offspring production (similar to

R_0) of a *D. magna* population from a Spanish temporary freshwater pond at 0.5 µg Cd/L was significantly higher than CV_G in the control. This is 38 times lower than the concentration of 18.9 µg Cd/L where we observed a significantly higher $CV_G(R_0)$. Barata et al. (2000b) found a significant increase of the CV_G from about 30% in the control to about 40% in a 0.5 µg Cd/L treatment. We found a significant increase from 22.4% in the control to about 71% in 18.9 µg Cd/L. While we found a reduction of the population mean of total reproduction of 92% at 18.9 µg Cd/L, Barata et al. (2000b) reported a reduction of about 30% at 0.5 µg Cd/L. Thus, the hypothesis proposed by Hoffmann et al. (1991), i.e. that increased stress (in this case Cd stress) is expected to result in increased genetic variation, is actually supported by the combined results of the present and the Barata et al. (2000b) study. The large difference in the populations' Cd sensitivity among both studies is more difficult to explain but can at least be partially explained by differences in Cd bioavailability. Indeed, both DOC (4 mg C/L) and hardness (250 mg $CaCO_3$/L) were higher in the present study than in the Barata et al. (2000b) study (no DOC added, 160 mg $CaCO_3$/L). In addition, the US-EPA equation for hardness correction of Cd ecotoxicity data (US-EPA, 2001) suggests a 1.5-fold lower toxicity in the present study compared to Barata et al. (2000b). Thus, the comparison made with Barata et al. (2000b) suggests that a potential micro-evolutionary response to Cd exposure (based on CV_G predictions) could be expected at very different concentrations in different populations. However, it is unlikely that bioavailability alone explains the 38-fold difference in increased micro-evolutionary potential. However, Barata et al. (2000b) did not account for maternal effects in the design of the study. Hence it is possible that their estimate of V_G included some maternal variance. Other factors such as differences in testing conditions (e.g. food quantity and quality, Heugens et al., 2006) may also have played a role. It would

therefore be recommended to perform additional studies with a range of *D. magna* populations from different habitats.

Additionally, differences in the evolutionary history of these two populations (prior to their collection in the field) may also have contributed to the sensitivity differences (in terms of population means). Indeed, different populations have different evolutionary histories and may develop different levels of stress sensitivity due to among-habitat differences of selective forces (Barata et al., 2002c). One such selective force may include the local Cd concentration, where it is expected that populations inhabiting higher Cd concentrations could have acquired increased tolerance (Ward and Robinson, 2005). Unfortunately, the history of the Cd concentrations in the two habitats considered in the Barata study (2000b) and our study, is unknown. Another important selective force impacting the genetic composition/structure of *Daphnia magna* populations is fish predation (De Meester et al., 1995), which was present in the population used in the present study but absent in the population used in the Barata et al. (2000b) study. It is interesting to speculate that different evolutionary histories with regard to the presence of a natural stressor (e.g, fish predation) could lead to among-population differences in the sensitivity of *D. magna* to Cd.

In summary, the use of measures of genetic variation of fitness under chemical stress, as determined in 21d-life table tests with *D. magna*, gives an indication at which concentrations a significant increase in the micro-evolutionary potential may take place (compared to the control) and hence, at which concentrations loss of clonal diversity may be enhanced. Research with more chemicals and more populations of *D. magna* and with other clonally propagating species is highly recommended. In addition, the micro-evolutionary potential under chemical stress (or of chemical tolerance) should also be determined to

species with other modes of reproduction. Chaumot et al. (2009), for instance, showed that the (narrow sense) heritability at an acutely toxic Cd concentration (20 µg Cd/L, resulting in median survival times of 12 hours to 7 days) in a natural population of the sexually reproducing *Gammarus fossarum* was not significantly different from zero, suggesting no potential for adaptation. Thus the micro-evolutionary potential to Cd stress (and chemical stress in general) is likely not only different within species (among populations) but also among species.

Finally, in our hypothesis 2, we stated that Cd could induce a cost of tolerance and that this would be reflected as a negative between-environment correlation of fitness between the control and the Cd environment (Table 2.2). A between-environment correlation indicates the extent to which the trait value of a genotype is proportional or not in two environments (Lynch et al., 1998). If this correlation equals one, it indicates that the genotype response is completely proportional in the two environments (Byers, 2005). Here, we did not find any negative correlations but rather only a positive correlation between the control and the Cd environment at 1.9 µg Cd/L, 4.0 µg Cd/L, 8.3 µg Cd/L for total reproduction, indicating that genotypes with higher fitness in the control environment generally also have a higher fitness in the Cd environment. For r_m, no significant correlations could be found. On the basis of these observations, hypothesis 2 was rejected: i.e. the existence of a cost of tolerance at (sub)lethal concentrations of Cd exposure for *D. magna* could not be demonstrated. This is in contrast with Agra et al. (2010), who indicated that in historically exposed *D. longispina* populations, acquired tolerance to Cu and Zn were inversely related with feeding rates in absence of the added metals. Also, Postma et al. (1995a) found a cost of tolerance in Cd-adapted *Chironomus riparius* populations when

reared in a clean environment. We suggest that the cost of tolerance that has been observed in more severely polluted environments for field populations (Medina et al., 2007; Postma et al., 1995; Agra et al., 2010) does not necessarily occur in mildly polluted environments, although based on results of this experiment, no cost of tolerance in a high Cd treatment (18.9 µg Cd/L) could be found.

5. Conclusion

This study suggests that increased Cd stress in a natural *D. magna* population results in a significant increased micro-evolutionary potential at 1.9 µg Cd/L (based only on $CV_G(r_m)$) and at 18.9 µg Cd/L (based on $CV_G(R_0)$, $CV_G(r_m)$, and $H^2(r_m)$). At these concentrations a stonger shift in genotypic composition on reduction of clonal diversity may be expected compared to the control. No negative between-environment correlations for r_m and total reproduction (R_0) between the control and the Cd environments were observed. This suggests that there is not necessarily a cost of tolerance whereby a population that adapts to Cd exposure would exhibit a reduced fitness after clean-up.

Chapter 3: The micro-evolutionary potential of *Daphnia magna* population exposed to temperature and cadmium stress.

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Abstract-This study examines micro-evolutionary aspects of a natural *Daphnia magna* population exposed to Cd. To this end, a set of hypotheses related to micro-evolutionary responses and to how these are influenced by temperature and Cd stress, were tested. Life-table experiments were conducted with 14 *D. magna* clones collected from an unpolluted lake following a 2×2 design with Cd concentration and temperature as the factors (control vs. 5 µg/L cadmium, 20°C vs. 24°C). Several fitness traits were monitored during 21 days. Our results demonstrate (1) that chemicals can have effects on key population genetic characteristics such as genetic variation and between-trait correlations and (2) that these effects may differ depending on temperature. These findings also suggests that further research is needed to understand the importance of combined chemical - global warming stress for micro-evolutionary responses of organisms. These aspects are currently not accounted for in any regulatory environmental risk assessment procedure.

1. Introduction

Ecotoxicology is predominantly concerned with assessing relatively short-term effects of chemicals on organisms (Van Straalen, 2003), i.e. effects occurring in a period usually no longer than one generation. As such effects of chemicals on phenotypic traits, such as survival, growth and reproduction, reported in literature mostly reflect the recent history of the individual, i.e. its initial response. Additionally, most ecotoxicity experiments are conducted in the laboratory with test populations with limited or even no genetic variation (e.g. monoclonal populations of *Daphnia* sp.) because this reduces variation and thus increases reproducibility of test results. Field populations, however, may be exposed to long-term chemical stress and do exhibit genetic variation. Both factors have mostly been ignored in routine ecotoxicology and environmental risk assessment. Field populations which experience an initially reduced fitness due to chemical exposure may exhibit phenotypic variation of fitness among individuals upon which natural selection may act. If this variation is also heritable - i.e. if it contains a significant genetic component - micro-evolutionary changes in the genetic make-up of the population may result in an increase of the mean fitness of the population. Thus, natural selection may result in genetic adaptation of a population to pollution (Medina et al., 2007; Lynch and Walsch, 1998).

Several studies have demonstrated the occurrence of genetic adaptation of populations to chemicals (or at least the potential for such adaptation) by examining the genetic variation and/or heritability of fitness traits. Barata et al. (2002a), for example, found significant genetic variation for cadmium tolerance within natural populations, suggesting a potential to acquire resistance to cadmium stress. As indicated in the previous chapter and also by other studies, increasing environmental stress enhances evolutionary rates by

increasing the expression of genetic variability in life history traits (Hoffmann and Parsons, 1991; Barata et al., 2000b). In general, knowledge of genetic adaptation to chemical stress is too limited to be accounted for in ecological risk assessment. In this chapter three aspects of micro-evolutionary responses to chemical exposure will be addressed. First, it has been shown that evolution can be constrained by genetic correlations among fitness-related traits (Reznick et al., 2000), also commonly referred to as trade-offs. A trade-off occurs when an increase in fitness due to a change in one fitness component is counter-acted by a decrease in fitness due to a concomitant change in another fitness component (Roff and Fairbairn, 2007). As such, analysis of genetic correlations does not only provide insight as to why evolution of fitness is constrained, but it can also indicate which of several individual fitness components may evolve along with fitness (due to natural selection) and in which direction. Although some data suggest that genetic correlations are dependent on environmental factors such as temperature and resource availability (Sgrò and Hoffmann, 2004), it has never been investigated if chemical exposure affects between-trait genetic correlation in aquatic organisms.

Second, it has been shown that populations adapted to contaminated environments may exhibit reduced fitness in unpolluted environments, an observation which is commonly referred to as “cost of tolerance” (Medina et al., 2007). This phenomenon is caused by genetic between-environment correlations or between-environment trade-offs (Medina et al., 2007). Shirley and Sibley (1999), for example, reported that a population of *Drosophila melanogaster* adapted to a high cadmium environment exhibited a lower fecundity in the absence of cadmium compared to a population adapted to a Cd-free environment. Although knowledge of between-environment trade-offs are considered a key element for

incorporating micro-evolution in the environmental risk assessment paradigm (Medina et al., 2007), this type of limited information is only available for highly contaminated environments (see reviews in Medina et al., 2007 and Morgan et al., 2007). Knowledge of such trade-offs in contaminated systems with low level and environmentally realistic chemical concentrations is completely lacking.

Third, although it has been shown that higher temperatures generally lead to increased toxicity of chemicals in conventional ecotoxicity experiments (e.g. Heugens et al., 2006), it has not been investigated what the effect of temperature is on the potential micro-evolutionary response of populations living under chemical stress. This type of information is relevant in the context of global warming.

The aims of the present study were to address these three aspects by testing a set of hypotheses related to (i) the micro-evolutionary potential, constraints and possible consequences for a natural population of *Daphnia magna* exposed to a sublethal cadmium concentration and (ii) the influence of temperature on these processes. The first hypothesis - as proposed by Barata et al. (2000b) - is that a population exposed to cadmium will exhibit more genetic variation for life-history traits than a control population (hypothesis 1). Second - following Heugens et al. (2003, 2006) - we hypothesize that an increased temperature in combination with cadmium exposure will lead to lower fitness (hypothesis 2). Following Hoffmann and Hercus (2000) this will in turn result in a higher genetic variability of life-history traits in a high temperature and high cadmium environment (hypothesis 3). Since genetic correlations may vary between environments (Sgrò and Hoffmann, 2004), we predict that cadmium exposure may alter genetic correlation between traits (hypothesis 4). Finally we hypothesize that there may be a cost of tolerance which will be reflected by a negative

genetic correlation of fitness between control and cadmium treatment in the two temperature environments (hypothesis 5).

To test these hypotheses, we conducted 21-day life-table experiments using a 2 × 2 design with Cd and temperature as experimental factors (control vs. 5 µg Cd/L, 20°C vs. 24°C). The Cd concentration of 5 µg/L was chosen because it was shown to lead to an inhibition of approximately 10% of reproductive output in three clones based on a preliminary experiment (see Supplementary material, Table S3.1). Since concentrations resulting in a 10% effect level (i.e. the EC_{10}) are commonly used as a basis for risk assessment or derivation of water quality criteria (e.g., EU, 2003) our choice enhances the regulatory relevance of the present investigation. Additionally, 5 µg Cd/L is within the range of Cd concentrations commonly reported in polluted water bodies, i.e. up to 28 µg Cd/L (Bervoets and Blust, 2003, Lopes et al., 2006).

All experiments were conducted with 14 different *D. magna* clones hatched from different ephippia, which had previously been collected from an unpolluted pond. Ephippial eggs are sexually-produced dormant eggs, that are representative of the genetic pool of a natural population and which can be used in the laboratory to study natural population responses (Barata et al., 2002a). Since *D. magna* also reproduce asexually by ameiotic parthenogenesis (Hebert, 1987), single genotypes can be tested in different environments, although it should be recognized that each genotype cultured and tested in the laboratory is a genotype that survived laboratory selection (Baird, 1992). By measuring different life-history traits of individuals of each genotype kept under different environmental conditions, estimates of quantitative genetic variation and genetic correlation between traits and

between environments were obtained as measures of the micro-evolutionary potential of the population (Barata et al., 2002b; Lynch and Walsh, 1998).

2. Materials and methods

2.1. General culture and exposure conditions

The maintenance of all clones of the natural population was performed as described in Chapter 2 (§2.1.).

2.2. Establishment and maintenance of clonal lineages of the natural population culture

Sediment containing *Daphnia* ephippia were collected from the Kasteelvijver pond in the nature reserve Blankaart (Diksmuide, Belgium) using a Van Veen grab and a sediment corer in October 2007. The samples were transferred to the laboratory and ephippia were isolated and hatched as described in Chapter 2 (§2.2.) A total of 14 randomly selected clonal lineages were used for all experiments.

2.3. Test design

2.3.1. Temperature acclimation

Organisms hatched from the ephippia were first acclimated to 20°C for two generations. A new generation was started with third or fourth brood offspring. The juveniles (<24h old) from a single clone were pooled and ten juveniles were randomly chosen from this pool to start a new generation of this lineage. Hence, each clone was presented in each generation by ten individual replicates maintained in polyethylene cups containing 50 mL of modified M4 medium. The juveniles of the second generation

acclimated to 20°C were used to start a third generation at 20°C and a first generation at 24°C. Each clone was acclimated for three additional generations to 24°C and to 20°C before starting the cadmium exposure experiment (see 2.3.2). The daphnids were fed daily with a 3:1 mixture (based on cell numbers) of the algae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* equivalent to 250 µg dry wt/*Daphnia*, 500 µg dry wt/*Daphnia* and 750 µg dry wt/*Daphnia* in the first, second and third week of their life, respectively. The test medium was renewed three times a week.

2.3.2. Cadmium exposure experiment

Based on a preliminary exposure experiment with five of the clones, a Cd concentration of 5 µg/L was selected as the sub-lethal test concentration in all Cd treatments (Supplementary material, Table S3.1). Cd exposures of all clones were simultaneously initiated at 20°C and 24°C with juveniles (<24h old) from the third or fourth brood of the temperature-acclimated adults. Ten juveniles of each clone at 20°C and 24°C were placed individually in 50 mL polyethylene cups with modified M4 medium and 5 µg Cd/L (added as $CdCl_2 \cdot H_2O$) and were subsequently monitored for 21 days following OECD test guideline No. 211 (OECD, 1998). Control exposures (no Cd added) at 20°C and 24°C with all clones were run in parallel.

Based on daily observations the following traits were determined: survival, time to first brood, length (of the parents) at first brood, reproduction (number of juveniles) at first brood, length (of the parents) at day 21 and total reproduction at day 21 (R_0). Population growth rate (r_m) was calculated as described in chapter 2 (§2.3, Eq.2.3).

The length of the parental organisms was defined as the linear distance between the top of the head and the base of the spine and was measured with the aid of a microscope (Kyowa, Tokio, Japan) equipped with a marked microscope slide (precision 0.1 mm) and using the Image Tool software (UTHSCSA, San Antonio, TX, USA).

Medium renewal and animal feeding were identical to that used in the temperature acclimation described above. Samples for analysis of dissolved Cd analysis were taken every week. pH, dissolved oxygen and temperature were measured at least twice a week and dissolved organic carbon (DOC) samples were taken at the beginning and end of the experiment.

2.4. Chemical analyses

Chemical analyses were performed as described in chapter 2 (§2.4.)

2.5. Statistical analyses

Genetic variation of total reproduction (R_0) and population growth rate (r_m) was compared among the different Cd treatments using the genotypic coefficient of variation (CV_G, Eq. 2.2) and broad sense heritability (H^2, Eq. 2.1), rather than the genetic variance (V_G) itself. For each Cd treatment, V_G, the environmental (or residual) variance (V_E) and confidence intervals were estimated as described in Chapter 2 (§2.5).

The median values (50th percentile) and the 2.5th and 97.5th percentile of CV_G, H^2 and $\overline{X}$ are reported. If more than 95% of the calculations yielded CV_G(Cd) > CV_G(control) or H^2(Cd) > H^2(control), the CV_G or H^2 in the Cd treatment was considered significantly higher than in the control. The population mean in a Cd treatment was considered significantly

lower than in the control if more than 95% of the calculations yielded $\overline{X}$ (control) > $\overline{X}$ (Cd) (i.e. equivalent to a one-sided test at the 0.05 significance level). All calculations were performed in MATLAB 7.5.0.342 (Mathworks Inc) software.

Genetic correlations between environments were calculated as described in chapter 2 (§2.5.). Genetic correlations between two traits, i.e. R_0 and r_m (within each environment) ($\rho_{G,trait1,trait2}$) were calculated as follows:

$$\rho_{G,trait1,trait2} = \frac{\mathrm{cov}\left(trait_1, trait_2\right)}{\sqrt{V_{G,trait1} \cdot V_{G,trait2}}} \quad \text{(Eq. 3.1)}$$

Where the variances of the two individual traits ($V_{G,trait1}$ and $V_{G,trait2}$) are calculated as above, and where cov($trait_1$,$trait_2$) is the covariance between traits 1 and 2 which is calculated with the method of the moments (cf. above), but now applied on cross-products of deviations (Lynch et al., 1998).

We considered a genetic correlation (between traits or between environments) significantly different from zero if ρ_G >0 in >95% of the calculations (positive correlation) or if ρ_G <0 in >95% of the calculations (negative correlation). Finally we compared genetic correlations between traits ($\rho_{G,trait1,trait2}$) obtained in the control vs. Cd environment. Correlations were considered significantly different if $\rho_{G,trait1,trait2}$ in the Cd exposure was higher than that in the control in >97.5% of the calculations or if it was lower in >97.5% of the calculations (i.e. equivalent to a two-sided test at the 0.05 significance level). All calculations were performed in MATLAB 7.5.0.342 (Mathworks Inc) software.

3. Results

3.1. Preliminary experiment

To select the Cd concentration to be used in our experiments, a preliminary 14 day chronic ecotoxicity test was conducted following OECD guideline No 211 (OECD, 1998) in our modified M4 medium. Three of the 14 *D. magna* clones were exposed at 20°C to a control and five concentrations ranging from 5 to 56 µg Cd/L. Ten replicates were used for each test concentration and each clone. Total reproductive output (number of juveniles/female) after 14 days was used as endpoint. The EC_{10} was calculated with the log-logistic model (Van Ewijk and Hoekstra, 1993) in Statistica 6 (Statsoft, Tulsa, OK). The EC_{10} values for the three clones ranged from 3.16 and 5.30 µg Cd/L. A nominal concentration of 5 µg Cd/L was selected for further testing because it was close to the EC_{10}s obtained for the three clones. Because the EC_{10} is commonly used as a basis for risk assessment or derivation of water quality criteria (e.g. EU, 2003) this choice enhances the regulatory relevance of the present investigation.

3.2. Chemical analyses

The results of the chemical analyses during the exposures are summarized in the Supplementary material (Table S3.2). Briefly, the cadmium concentrations in the control treatments were always below the detection limit of 30 ng Cd/L. The mean cadmium concentration (after filtering) of the freshly prepared medium was the same in the 20°C+Cd and the 24°C + Cd treatment, i.e. 4.3 µg Cd/L, which is within 15% of the nominal concentration of 5 µg/L. DOC concentrations during the test ranged from 3.7 to 5.7 mg C/L and were similar in the four treatments. The pH ranged between 7.3 and 7.6 and was also similar in the four environments.

3.3. Population means

Values of traits for all individuals, all clones and all environments are given in Supplementary material, Table S3.3 – Table S3.26. Population means of all traits in the four treatments are reported in Table 3.1. At 20°C, *D. magna* exposed to cadmium exhibited, compared to the control, a significantly lower number of offspring in the first brood (-11%), lower reproduction during 21 days (R_0) (-12%) and lower length at day 21 (-4%). Time to first brood was significantly higher (+3%) in the cadmium treatment. Other traits were not significantly affected. Cd had a similar but greater effect at 24°C, i.e. reproduction at first brood (-20%), reproduction during 21 days (-20%) and length at day 21 (-5%). Length at first brood (-4%) and population growth rate (-7%) were also significantly affected.

Table 3.1: Median of simulated population means (=mean of clone means) of the fitness traits. Numbers between brackets represent the 95% confidence interval. An asterisk (*) indicates a significant ($p<0.05$) difference between the control group and the Cd treatment within one temperature treatment (20°C and 24°C). Numbers between parentheses indicate the % difference between the Cd and the control treatment.

Fitness trait	20 °C (Control)	20°C and Cd	24°C (Control)	24°C and Cd
r_m	0.41 [0.40-041]	0.40 [0.39-0.41] (-3.0%)	0.46 [0.44-0.47]	0.43 [0.42-0.44]* (-7.0%)
Reproduction at first brood	13.91 [13.16-14.70]	12.35 [11.54-13.16]* (-11%)	11.80 [11.02-12.57]	9.45 [8.84-10.02]* (-20%)
Length at first brood (mm)	3.10 [3.06-3.14]	3.06 [3.01-3.10] (-2.0%)	2.97 [2.94-3.00]	2.87 [2.83-2.91]* (-4.0%)
Time to first brood (days)	7.46 [7.30-7.63]	7.66 [7.46-7.88]* (+3%)	6.38 [6.23-6.53]	6.31 [6.19-6.45] (-1.0%)
Total reproduction (R_0)	111.75 [104.16-122.07]	98.76 [91.37-104.36]* (-12%)	119.22 [112.76-126.95]	95.93 [87.16-104.20]* (-20%)
Length at day 21 (mm)	4.16 [4.10-4.22]	4.03 [3.96-4.11]* (-4.0%)	4.13 [4.08-4.17]	3.92 [3.84-4.00]* (-5.0%)

3.4. Genetic coefficients of variation and broad sense heritabilities

Genetic coefficients of variation (CV_G) of the different traits are shown in Figure 3.1. The CV_G for all traits and all treatments were significantly greater than 0, indicating that there is significant genetic variation for all traits in all treatments. Overall, CV_G was highest for total reproduction (i.e. from 13.1% to 19.4%) and reproduction at the first brood (i.e. from 11.4% to 13.6 %) followed by time to first brood (i.e. from 3.7% to 6.1%) and r_m (i.e. from 4.2% to 5.9%). It was lowest for length at first brood (i.e. from 1.7% to 3.1%) and length at day 21 (1.6% to 4.5%). We also observed a significant increase in CV_G (compared to

control) in the Cd treatment at 24°C for the following fitness traits: length at first brood, length at day 21 and total reproduction. At 20°C, the CV_G of none of the traits was affected by the cadmium treatment.

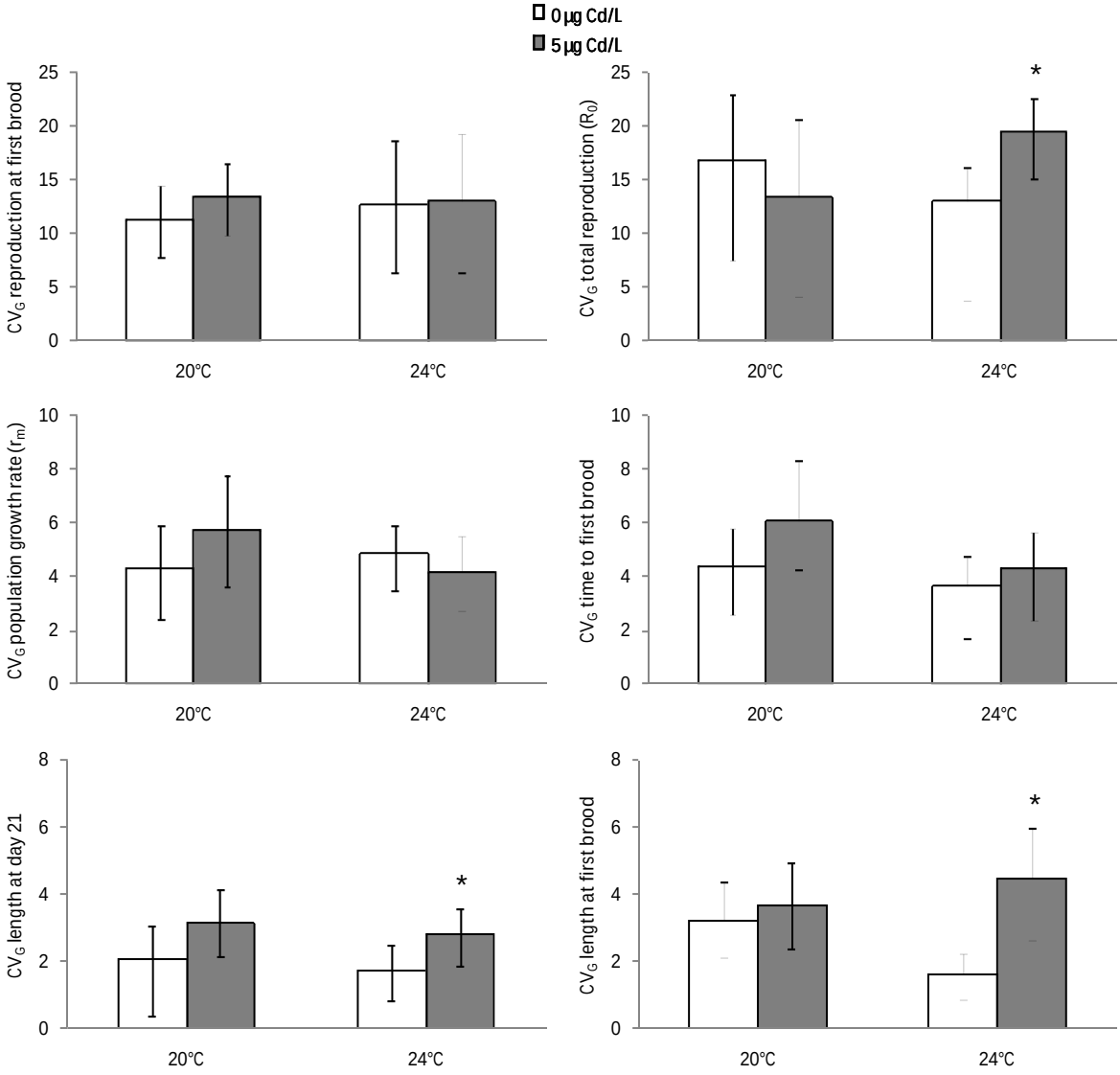

Figure 3.1: Median genetic coefficients of variation (CV_G, %) for different fitness traits in a *D. magna* population. Error bars represent the 95% confidence intervals. An asterisk (*) indicates a significant difference between the Cd and the control treatment within a temperature treatment ($p<0.05$).

Broad sense heritabilities (H^2) of the different traits are shown in Figure 3.2. The H^2 for all traits and all treatments were significantly greater than 0, indicating that there is significant genetic variation for all traits in all treatments. H^2 was similar for total

reproduction (i.e. from 0.39 to 0.58) and population growth rate (i.e. from 0.35 to 0.58), followed by length at day 21 (i.e. from 0.12 to 0.56). It was lowest for reproduction at first brood (i.e. from 0.26 to 0.37), time to first brood (i.e. from 0.13 to 0.49) and length at first brood (i.e. from 0.13 to 0.40). We observed a significant increase in H^2 (compared to control) in the Cd treatment at 20°C for length at first brood. At 24°C, the H^2 of length at first brood and lenght at day 21 were affected by the cadmium treatment.

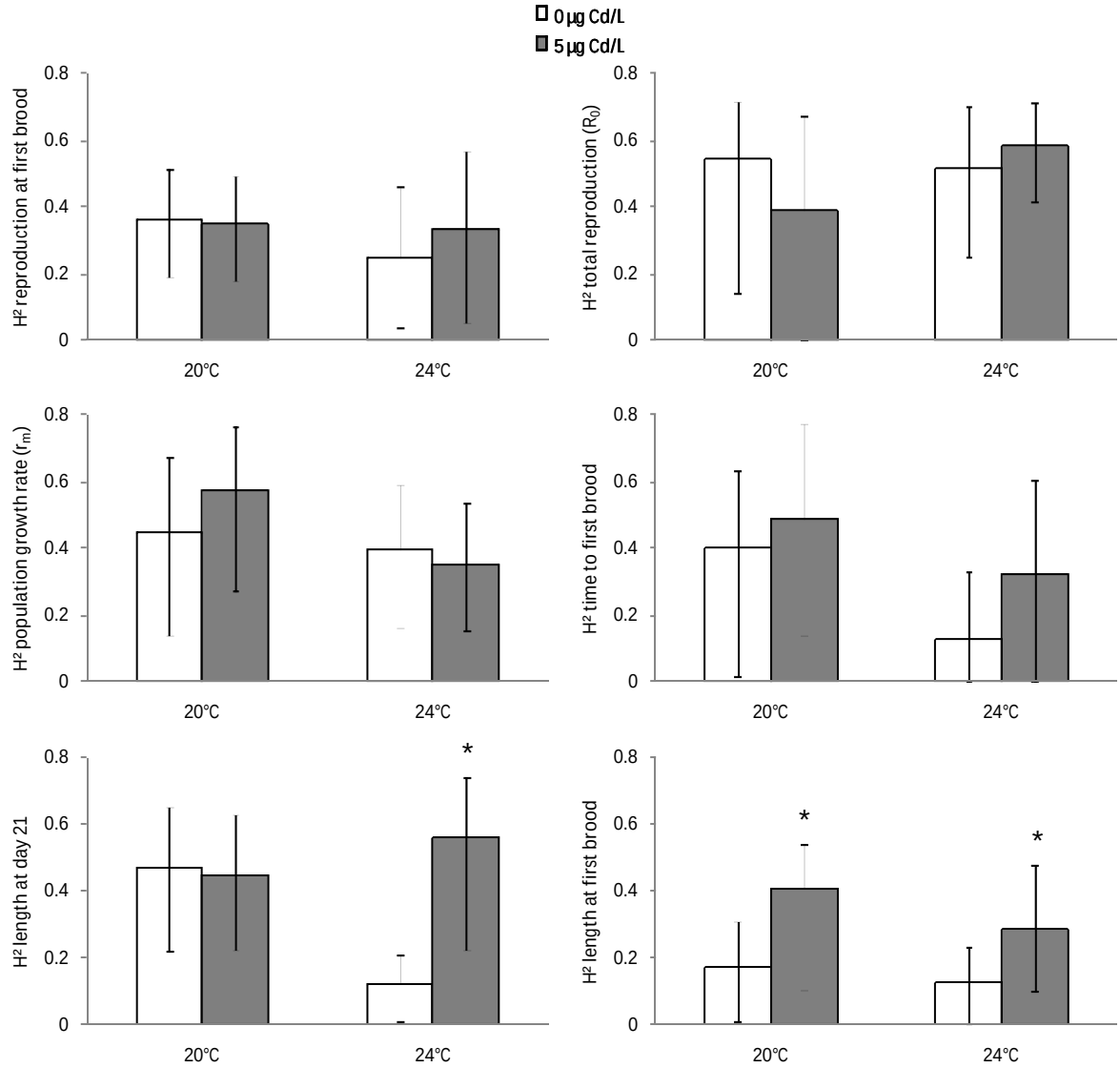

Figure 3.2: Median Broad sense heritability (H^2) for different fitness traits in a *D. magna* population. Error bars represent the 95% confidence intervals. An asterisk (*) indicates a significant difference between the Cd and the control treatment within a temperature treatment ($p<0.05$).

3.5. Genetic correlations between traits

A comprehensive overview of genetic correlations between all traits in the four environments is presented in Table 3.2 - 3.5. Correlations between r_m and traits related to the first brood are also shown in Figure 3.3.

At 20 °C, five significant between-trait correlations were found in the control and the Cd treatment. Three were observed in both treatments: (1) a negative correlation between r_m and time to first brood, (2) a positive correlation between length at first brood and reproduction at first brood and (3) a positive correlation between length at first brood and length at day 21. Two significant correlations were observed only in the control at 20°C: (1) a positive correlation between r_m and length at first brood, and (2) a negative correlation between length at first brood and time to first brood. Two significant correlations were observed only in the Cd treatment at 20°C: (1) a positive correlation between r_m and length at day 21, and (2) a negative correlation between length at day 21 and time to first brood.

Table 3.2: Median genetic correlations between fitness traits ($\rho_{trait1,trati2}$) at 20°C and no Cd added (Control). Numbers between brackets represent the 5th and 95th percentile genetic correlation coefficient between traits. An asterisk (*) indicates a significant between-trait correlation that is significant ($p<0.05$).

	Reproduction at first brood	Length at first brood	Time to first brood	R_0	Length at day 21
r_m	0.48 [-0.35 , 0.89]	0.79* [0.18 , 1.35]	-0.73* [-0.90 , -0.21]	0.25 [-0.25 , 0.90]	0.25 [-0.16 , 0.70]
Reproduction at first brood		1.06* [0.90 , 1.36]	-0.26 [-0.69 , 0.34]	-0.03 [-0.46 , 0.45]	0.14 [-0.44 , 0.69]
Length at first brood			-0.50* [-1.07 , -0.04]	0.13 [-0.41 , 1.04]	0.75* [0.30 , 1.26]
Time to first brood				0.22 [-0.57 , 0.79]	-0.35 [-0.81 , 0.14]
R_0					-0.05 [-0.54 , 0.70]

Table 3.3: Median genetic correlations between fitness traits ($\rho_{trait1,trati2}$) at 20°C and 5 µg Cd/L. Numbers between brackets represent the 5th and 95th percentile genetic correlation coefficient between traits. An asterisk (*) indicates a significant between-trait correlation that is significant ($p<0.05$).

	Reproduction at first brood	Length at first brood	Time to first brood	R_0	Length at day 21
r_m	0.47 [-0.15 , 0.84]	0.32 [-0.16 , 0.67]	-0.88* [-0.99, -0.56]	0.14 [-0.41 , 1.07]	0.61* [0.37 , 0.86]
Reproduction at first brood		0.55* [0.12 , 0.93]	-0.44 [-0.85 , 0.23]	-0.06 [-0.62 , 0.92]	0.43 [-0.01 , 0.76]
Length at First brood			-0.35 [-0.70 , 0.10]	-0.34 [-0.86 , 0.85]	0.71* [0.25 , 0.97]
Time to first brood				-0.39 [-1.33, 0.02]	-0.53* [-0.96 , -0.10]
R_0					0.21 [-0.14 , 0.94]

At 24 °C, fewer significant correlations were found. In the control, a positive correlation was found between R and length at day 21. In the Cd treatment, three positive correlations were noted: (1) between r_m and reproduction at first brood, (2) between length at first brood and reproduction at first brood and (3) between length at day 21 and length at first brood.

Table 3.4: Median genetic correlations between fitness traits ($\rho_{trait1,trait2}$) at 24°C and no Cd added (control). Numbers between brackets represent the 5th and 95th percentile genetic correlation coefficient between traits. An asterisk (*) indicates a significant between-trait correlation that is significant ($p<0.05$). The underlined value indicates that the correlation is significantly different between the control and the Cd treatment at 24°C ($p< 0.05$).

	Reproduction at first brood	Length at first brood	Time to first brood	R_0	Length at day 21
r_m	0.55 [-0.01, 0.92]	0.29 [-0.34 , 0.95]	-0.68 [-0.90 , 0.00]	0.12 [-0.45 , 0.67]	0.30 [-0.37 , 0.85]
Reproduction at first brood		<u>0.00</u> [-1.09 , 0.76]	-0.01 [-0.76 , 0.74]	-0.45 [-0.80 , 0.07]	-0.65 [-1.34 , 0.14]
Length at first brood			-0.79 [-1.71 , 0.09]	-0.25 [-0.82 , 0.38]	0.74 [-0.22 , 1.51]
Time to first brood				0.23 [-0.52 , 0.98]	-0.63 [-1.47 , 0.32]
R_0					0.67* [0.03, 1.22]

Table 3.5: Median genetic correlations between fitness traits ($\rho_{trait1,trait2}$) at 24°C and 5 µg Cd/L. Numbers between brackets represent the 5th and 95th percentile genetic correlation coefficient between traits. An asterisk (*) indicates a significant between-trait correlation that is significant ($p<0.05$). The underlined value indicates that the correlation is significantly different between the control and the Cd treatment at 24°C ($p< 0.05$).

	Reproduction at first brood	Length at first brood	Time to first brood	R_0	Length at day 21
r_m	0.60* [0.02 , 0.86]	0.18 [-0.73 , 0.77]	-0.39 [-0.88 , 0.19]	-0.07 [-0.71 , 0.51]	-0.08 [-0.70 , 0.83]
Reproduction at first brood		<u>0.89*</u> [0.39 , 1.31]	0.06 [-0.43 , 0.44]	-0.06 [-0.68 , 0.63]	0.31 [-0.38 , 0.76]
Length at first brood			0.21 [-0.43, 0.78]	0.17 [-0.47 , 0.94]	0.74* [0.38 , 0.95]
Time to first brood				0.62 [0.00 , 0.97]	0.56 [-0.08 , 1.18]
R_0					0.52 [-0.00 , 0.81]

Although these data suggest that different patterns of between-trait correlations exist between the control and Cd treatments, direct statistical comparison of between-trait correlations reveals that only the correlation between length at first brood and reproduction at first brood is statistically different between the control and the Cd treatment.

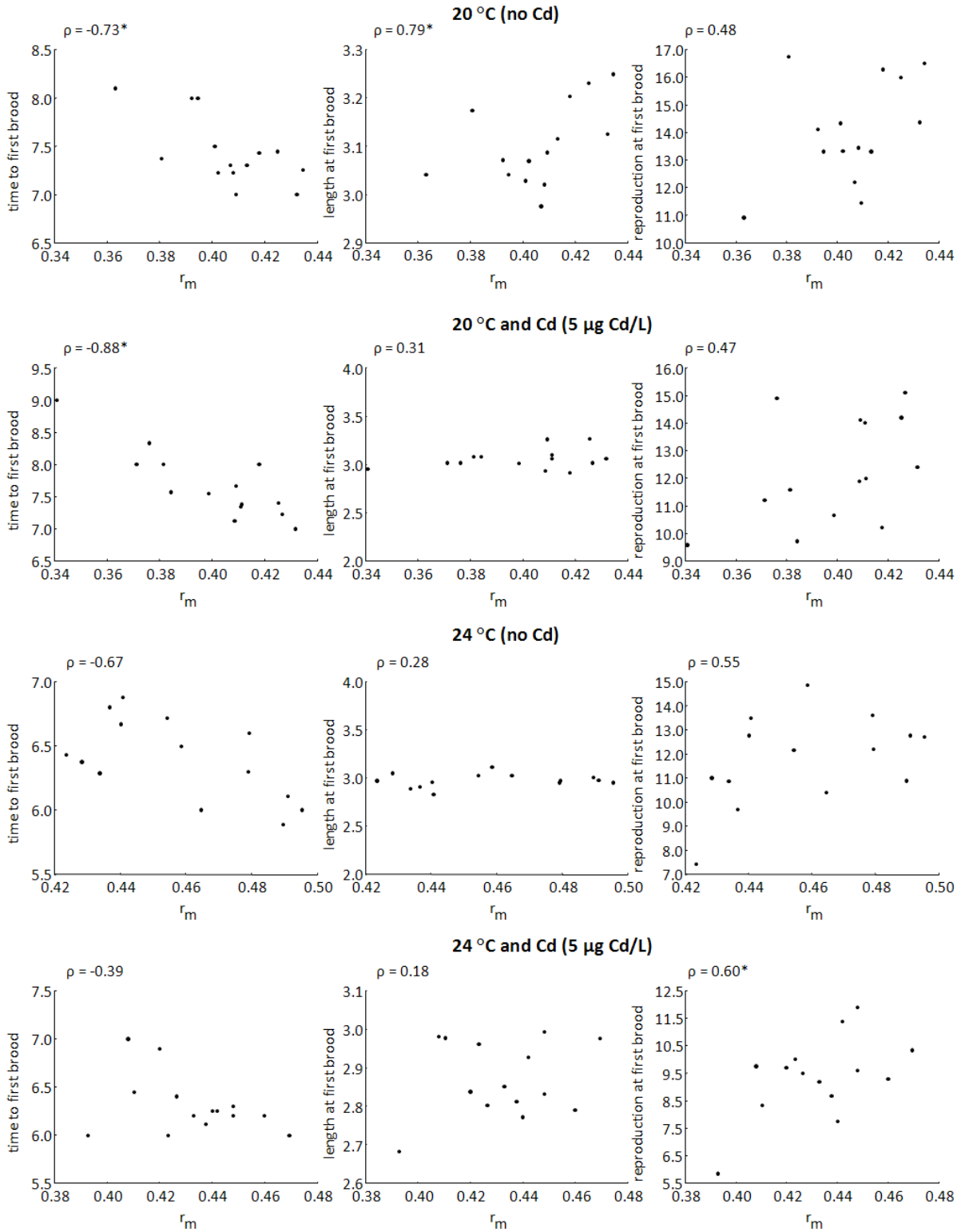

Figure 3.3: Genetic correlations between r_m (population growth rate) and time to first brood (left), length at first brood (middle) and reproduction at first brood (right) in the four different treatments (top to bottom). Each dot represents the mean trait value of a clone. The median genetic correlation is given at the top of each graph and can also be found in Tables 3.2-3.5. An asterisk (*) indicates that the correlation is significant ($p<0.05$).

3.6. Genetic correlation between environments

We found a significant positive between-environment genetic correlation for r_m between the 20°C control and the 20°C + Cd treatment (ρ = 0.77). At 24°C, this correlation was not significant (ρ = 0.32) (Figure 3.4). The 5th and 95th genetic correlation coefficient are given in Supplementary material, Table S27.

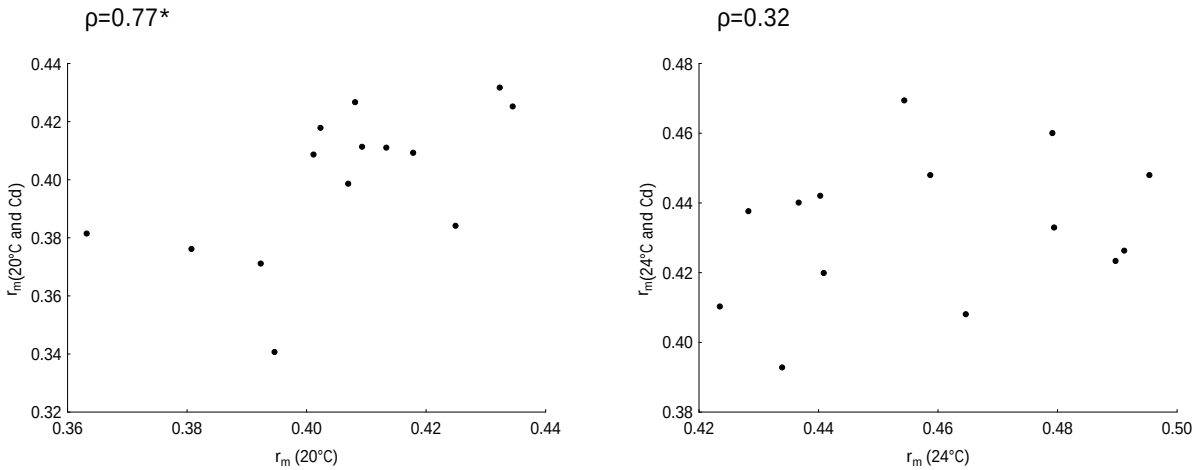

Figure 3.4: Genetic correlations between the control and the cadmium environment for r_m (population growth rate) at 20°C (left) and at 24°C (right). Each dot represents the mean r_m for each clone. An asterisk (*) indicates that the correlation is significant (p<0.05).

4. Discussion

This present study likes to address three aspects of micro-evolutionary potential in a natural population of *Daphnia magna* exposed to a sublethal Cd contamination and combined with the effects of temperature.

Studies with various species suggest that environmental stress may increase evolutionary rates by increasing the level of genetic variability in life-history traits (Hoffmann and Parsons, 1991; Barata et al., 2002 a,b). In this context, Houle (1992) suggested that for comparative purposes the genetic coefficient of variation (CV_G) is a better measure of genetic variability than heritability. This coefficient is also more closely related to the

response of fitness traits to natural selection (Houle, 1992) although in this study, also H^2 was determined. A higher CV_G and/or H^2 thus suggests a higher evolutionary potential. Our results show that all traits exhibited a significant CV_G and H^2 in both control and Cd environments which indicates that under all conditions tested there is evolutionary potential of the population for all of these traits. This is in contrast with the previous chapter, where only for R_0, at control and at 4.0 µg Cd/L (similar to 4.3 µg Cd/L in this study) a significant CV_G and H^2 was found. It also has to be noted as described in the previous chapter that the CV_G and H^2 determined in the present study represent total genetic variation based on interclonal variation (i.e. the sum of the additive, epistatis and dominance components of genetic variation) and that the additive genetic variation and narrow sense heritability would probably be a more precise estimate of evolutionary potential (with total genetic variation being an upper limit of the additive variation).

The CV_G and H^2 can also be used to test the hypothesis whether the Cd exposed population exhibits more genetic variation for life-history traits than the control population (hypothesis 1). For H^2, at 20°C a significant effect was found for length at day 21, but this hypothesis was rejected for CV_G as there was no significant effect of exposure to 4.3 µg Cd/L (actual Cd concentration) on the CV_G for any of the traits. This appears to be in contrast with the findings of Barata et al. (2000b) who found a significant increase of the CV_G from about 30% in the control to about 40% in a 0.5 µg Cd/L treatment. Although the Cd concentration in Barata et al. (2000b) is nine times lower than that used in our study, the Cd-induced stress was clearly higher in the Barata et al. (2000b) study. Indeed, while we found a reduction of the population mean of the reproduction at first brood of only 11% at 4.3 µg Cd/L, Barata et al. (2000b) reported a reduction of about 30% at 0.5 µg Cd/L. Thus, the hypothesis proposed

by Hoffmann and Parsons (1991), i.e. that increased stress (in this case Cd stress) is expected to result in increased genetic variation, is actually supported by the combined results of the present and the Barata et al. (2000b) study. Bio-availability can partially explain the found discrepancies between the two studies. Indeed, both DOC (4 mg C/L) and hardness (250 mg $CaCO_3$/L) were higher in the present study than in the Barata et al. (2000) study (no DOC added, 160 mg $CaCO_3$/L). Speciation calculations using WHAMVI (Tipping, 1994) indicated that in our study 65% of the dissolved Cd was present as free ionic Cd^{2+}, while 93% was Cd^{2+} in the Barata et al. (2000) study. Thus binding of Cd to DOC would explain part of the observed 'sensitivity' difference. In addition, the US-EPA equation for hardness correction of Cd ecotoxicity data (US-EPA, 2001) suggests a 1.5-fold lower toxicity in the present study compared to Barata et al. (2000). However, it is unlikely that bioavailability alone explains the nine-fold difference in toxicity. Additionally, as described in the previous chapter, differences in food regimes during tests and differences in the evolutionary history of these two populations (prior to their collection in the field) may also have contributed to the sensitivity differences.

Compared to the 20°C treatment in our study, the adverse effects of Cd at 24°C on the *D. magna* population were more pronounced. Not only were more traits affected, they were also affected to a larger extent (see Table 3.1). This is in accordance with Heugens et al. (2003, 2006) and supports our second hypothesis. Increased toxicity at higher temperature is most often attributed to increased physical rates in ectotherms, including chemical uptake rates (Heugens et al., 2006). Although uptake rates of Cd were not measured here, the increased metabolic rate at 24°C is apparent from the faster maturation (time to first brood) at 24°C (Table 3.1). Comparison of the CV_G values obtained in the controls to those in the Cd

treatment at 24°C reveals an increased CV_G in the Cd treatment for length at first brood, length at day 21 and R_0. For H^2, a significant increase was found for length at first brood and length at day 21. Higher temperature (24°C) thus lead to an increase in the Cd stress (compared to the Cd stress at 20°C) which in turn resulted in a significant increase in CV_G and/or H^2 for some traits. This observation supports hypothesis 3. Indeed, our data shows that an increase in temperature does not only increase the magnitude of Cd stress, but also increases the expression of genetic variation of some traits. This in turn may increase the micro-evolutionary potential under Cd stress and may eventually lead to shifts in genotype frequencies within a population. If this pattern is confirmed for more substances, the combination of e.g. global warming with chemical exposure may increase selection intensity in natural populations and increase the likelihood of genetic erosion (Van Straalen and Timmermans, 2002). It must be kept in mind though that the three traits exhibiting higher CV_G and/or H^2 in the 24°C + Cd treatment (length at first brood, length at day 21 and R_0) are usually considered not to be those with the closest relation to actual fitness in the field. Both timing and clutch size of the first brood are more important in this regard (Barata et al., 2002a) and it has been shown that these traits can be under selection in stressful environments (Lopes et al., 2004). In general, r_m - which is largely determined by timing and clutch size of the first (few) broods - is generally considered an integrative measure of fitness with a predictive capacity of actual fitness in the field (Calow et al., 1997; Hooper et al., 2008). However, no significant differences in CV_G and H^2 between control and Cd treatments were observed for r_m. The CV_G and H^2 for r_m is, however, significantly higher than zero in all treatments suggesting that natural selection may occur and traits associated with r_m may also evolve.

The extent to which two traits are genetically associated can be determined by their genetic correlation. Genetic correlations between traits can arise due to pleiotropy or linkage, but no distinction between these two mechanisms can be made in the present study. Differences of between-trait correlation among environments suggest different micro-evolutionary paths across environments (Byers, 2005). Our results (Tables 3.2 - 3.5) show that significant correlation between traits do exist in *D. magna* and suggest that the patterns of between-trait correlation are different among the four environments investigated here. The r_m in the 20°C control treatment was positively correlated with length at first brood and negatively correlated with time to first brood (Figure 3.3, Table 3.2). This result should be interpreted with caution as the latter trait is mathematically related to r_m (i.e. shorter time to first brood results in higher calculated values of r_m (Eq.2.1)). Thus natural selection is predicted to favour genotypes with faster maturation *because* these genotypes have the highest fitness (i.e. highest r_m). On the other hand, selection is predicted to result in a population with increased mean length at first brood (*as a consequence of* the positive correlation with r_m). In the 20°C plus Cd treatment, the genotypes with the largest r_m were those with the shortest time to first brood and highest length at day 21, but no correlation between r_m and length at first brood was observed. Under these conditions, natural selection - which naturally favours genotypes with highest r_m - is predicted to favour genotypes with a larger size (at day 21) and faster maturation. This observation is similar to the results obtained with *Drosophila melanogaster*, for which selection for increased Cd tolerance resulted in increased fecundity and decreased developmental time (Shirley and Sibley, 1999). In the 24°C control treatment no significant correlations between r_m and any other trait were noted. Organisms in the 24°C plus Cd treatment did exhibit a positive correlation between r_m and reproduction at first brood. Here, natural selection under Cd

stress is predicted to result in a population with a higher reproduction at first brood, which is a different micro-evolutionary path as predicted for organisms exposed to the 20°C plus Cd treatment (see above). Overall, the between-trait correlation data of both the 20°C and 24°C treatments suggest that different genetic correlations between traits are observed in the control vs. the Cd environment. This supports our fourth hypothesis: i.e. that Cd affects between-trait correlations. However, this latter conclusion should be treated with caution since a direct statistical comparison could not detect significant differences in between-trait correlation between the control and the Cd environment at 20°C. Also at 24°C only a single difference, i.e. for the correlation between length at first brood and reproduction at first brood (Tables 3.4 - 3.5), was noted. This lack of significance was unexpected since in some cases relatively large differences of the estimated median values of these correlations were observed (Tables 3.2 - 3.5). Closer examination of the bootstrap calculation output revealed that the between-trait correlations had relatively large confidence intervals. Hence future experiments would probably benefit from increasing the sample size (i.e. increasing number of clones). We conducted a power analysis and this showed that in order to detect absolute differences in $\rho_{trait1,trait2}$ values (among environments) equal to 0.68 and 0.32 with a power of 80%, sample sizes (number of clones) of 60 and 200 would be needed, respectively (see Supplementary material S3.28 for more details). This is considerably higher than the 14 clones used in the present study. With 14 clones, the absolute difference in $\rho_{trait1,trait2}$ values (among environments) would have to be as high as 1.2, which explains why we detected few differences as statistically significant in the present study.

Finally, in our hypothesis 5, we stated that Cd could induce a cost of tolerance and that this would be reflected in a negative between-environment correlation of fitness

between the control and the Cd environment (Figure 3.3). Here, we did not find a negative correlation but rather a positive correlation between the control and the Cd environment (Figure 3.3) at 20°C, indicating that genotypes with higher fitness in the control environment generally also have a higher fitness in the Cd environment (positive correlations were also found in chapter 2 for total reproduction (R_0)). At 24°C this genetic correlation was not significant. On the basis of these observations, hypothesis 5 was rejected: i.e. the existence of a cost of tolerance at sublethal concentrations of Cd exposure for *D. magna* could not be demonstrated. We suggest that the cost of tolerance that has been observed in more severely polluted environments (Medina et al., 2007; Postma et al., 1995; Agra et al., 2010) does not necessarily occur in mildly polluted environments, although based on results of the previous chapter, no cost of tolerance in a high Cd treatment could be found.

5. Conclusion

We have examined some aspects of micro-evolutionary potential following Cd exposure of *D. magna* that have not been previously studied and the following conclusions are proposed. First, temperature increase (e.g. possibly due to global warming) may impact the way Cd affects genetic variation. Second, sub-lethal Cd concentrations have the potential to modify the genetic correlations between traits and the direction in which exposed population's traits may evolve. While this is a first indication that Cd exposure may affect micro-evolutionary paths of *D. magna* populations, stronger statistical evidence for this statement is needed because current sample sizes resulted in relatively large confidence intervals on estimates of between-trait correlations. Third, no negative between-environment correlations for r_m between the control and the Cd environment were observed. This suggests that there is not necessarily a cost of tolerance whereby a

population that adapts to sublethal Cd exposure would exhibit a reduced fitness if returned to a non Cd-polluted environment. Finally, our results suggest that chemicals can have effects on fundamental population genetic characteristics such as genetic variation and between-trait correlations and that these effects may differ depending on temperature. They also suggest that chemical exposure combined with global warming may result in micro-evolutionary responses of populations that are currently not accounted for in any environmental risk assessment procedure.

Chapter 4: Potential for adaptation in a *Daphnia magna* population: additive and non-additive components under Cd and temperature stress

Abstract- The genetic variability within a population determines the micro-evolutionary potential of a population exposed to stress. As this genetic variability may also contain a non-additive genetic component, the total genetic variability could overestimate this potential for adaptation. In this study, we examined the additive and non-additive components of the genetic variability of fitness traits in a natural *Daphnia magna* population exposed to Cd and temperature stress. Life-table experiments were conducted with 20 parent and 39 offspring clones following a 2×2 design with Cd concentration and temperature as the factors (control vs. 5 µg/L cadmium, 20°C vs. 24°C). Total reproduction (R_0) and population growth rate (r_m) were determined. Variance components were determined using an Animal Model. Narrow sense heritability (h^2) and the additive genetic coefficient of variation (CV_A) of total reproduction (R_0) ranged between 0.03 and 0.22 and between 9% and 82% respectively. CV_A and h^2 were significantly >0 in the 24°C + Cd treatment. A significant Cd effect on h^2 and CV_A was observed at 24°C (compared to the control). For total reproduction a significantly >0 additive genetic variance was detected in 24°C + Cd. The additive and dominance components of variation of population growth rate (r_m) could not be estimated at 20°C, because of missing data and too low variation. At 24°C, no significant additive and dominance components were found in the control and Cd treatment for r_m. h^2 ranged between 0.04 and 0.13, and CV_A between 11.3% and 11.7% for population growth rate (r_m). Our results indicate that temperature and Cd can have significant effects on additive and non-additive components of genetic variability of fitness traits in *D. magna* population. The finding of a significant additive genetic variance of fitness in the 24°C + Cd treatment indicates that genetically determined differences of fitness among clones under Cd stress may be heritable to the next generations.

1. Introduction

Conventional risk assessment is usually based on short-term effects of chemicals on populations with limited genetic variation (i.e. effects occurring in a period no longer than one generation). However, natural populations usually consist of a large number of genetically different individuals, which may respond differently to stress. Long-term (i.e. multi-generational) exposure to contaminants can lead to micro-evolutionary changes in a population, which could lead to an increase of tolerance to the contaminant of interest (i.e. adaptation). These micro-evolutionary changes can be: (1) the disappearance of the sensitive individuals (genetic erosion) or (2) the appearance of new alleles conferring tolerance (through mutations) or (3) the appearance of a combination of genes underlying a new or more efficient tolerance mechanism followed by their increased frequency by natural selection (Lopes et al., 2006). Although some studies reported increased tolerance in aquatic populations following long-term exposure to chemicals (Xie et al., 2003; Ward and Robinson, 2005), genetic adaptation appears to be infrequent according to other studies (Klerks, 2002; Chaumot et al., 2009).

The micro-evolutionary potential for adaptation can be quantified by using multi-generation artificial selection experiments or through identification of genetically determined differences in tolerance within a population (standing genetic variation). Several studies with *Daphnia sp.* clones have indicated the existence of such a significant genetic variation in tolerance within a population for metals (Baird et al., 1990; Barata et al. 1998; Barata et al. 2000; Barata et al. 2002a,b,c; Messiaen et al., 2010). Messiaen et al. (2010) indicated that an increase of temperature (24°C versus 20°C) led to an increased expression of the genetic variability of some fitness traits in a natural *D. magna* population under Cd

stress (compared to a control, unstressed situation). Under long-term, multi-generation exposure of this population, this may eventually lead to stronger shifts in genotype frequencies within a population (compared to a control, unstressed conditions). In all above-cited studies, however, genetic variation has been assessed with a clonal approach. However, *Daphnia sp.* typically reproduce by cyclic parthenogenesis, which is an alteration between clonal reproduction under beneficial conditions and sexual reproduction when cued by the environment. Sexual reproduction results in the production of diapausing eggs that are encased in a desiccation-resistant ephippium. The above mentioned (clonal) estimations of total genetic variability (and also broad sense heritability and genetic coefficient of variation) also contain non-additive genetic interactions (Falconer and Mackay, 1996), which are not heritable across sexual generations, only the additive component is heritable. For the commonly used *Daphnia magna*, two studies estimated the h^2 under food stress (Ebert et al., 1993) and phototactic behavior (De Meester et al., 1991). The heritabilities of clutch size and adulth length were much larger in high than in low food (Ebert et al., 1993). There was a significant contribution of the additive component to the total phenotypic variance found in the heritability of phototactic behavior (De Meester al., 1991). The estimation of the additive component of genetic variation in a ecotoxicological context has until now hardly been explored in aquatic ecotoxicology (Klerks and Moureau, 2001; Chaumot et al., 2009). Very low h^2 (0-0.2) were found in the study of Klerks and Moureau (2001), who exposed adults and fry of sheephead minnow (*Cyprinodon variegatus*) to Zn, phenanthrene and mixtures of Zn, phenanthrene, Ni, Barium and three polycyclic aromatic hydrocarbons. Chaumot et al. (2009) found that the genetic differences in survival time in a *Gammarus fossarum* population exposed to lethal Cd stress (20 µg Cd/L) were explained by large non-additive variance components. As a consequence, the narrow sense

heritability was negligible. Chaumot et al. (2009) therefore postulated (based on weakness of additive components for Cd tolerance) that exceptional cases of adaptation of field populations would be permitted only by the fixation of rare alleles (Woods and Hoffmann, 2000). Nonetheless, quantitative genetic studies can provide more insight to the issue of adaptation in risk assessment (Chaumot et al., 2009).

The minimum requirements for the selection on a phenotypic trait in a population are (i) the presence of genetically heritable variation of that trait, (ii) the occurrence of natural selection and (iii) a genetic correlation of that trait with fitness (Lynch and Walsch, 1998; Templeton, 2006; Hoffmann and Hercus, 2000; Chaumot et al., 2009). In this study, we will investigate one of these requirements, i.e. the presence of genetically heritable variation. More specifically, the aim of this study was to determine: (1) the additive genetic variance under Cd stress, which indicates a heritable variation to the next generation in a *Daphnia magna* population (compared to a control, unstressed condition) and (2) the influence of temperature on the additive and non-additive genetic variance, heritabilities and coefficients of variation under Cd stress (compared to a control).

We performed a 21-day life table experiment with 20 genetically distinct clonal lineages established from ephippial eggs from a single natural *Daphnia magna* population and 39 sexual offspring clonal lineages under a control treatment (no Cd) and Cd treatment (5 µg Cd/L) at two temperatures (20°C versus 24°C). This allowed us to determine the additive and dominance components of genetic variance of two fitness traits, i.e. R_0 and r_m, under those treatments, as a proxy of the micro-evolutionary potential.

2. Material and Method

2.1. General culture and exposure conditions

The maintenance of all clones of the natural population was performed as described in Chapter 2 (§2.1.).

2.2. Establishment and maintenance of clonal lineages of the natural population culture

Sediment containing *Daphnia* ephippia were collected from the Kasteelvijver pond in the nature reserve Blankaart (Diksmuide, Belgium) using a Van Veen grab and a sediment corer in October 2007. The samples were transferred to the laboratory and ephippia were isolated and hatched as described in Chapter 2 (§2.2.) A total of 20 randomly selected clonal lineages were used as parental clones for all experiments (i.e. induction of males, crossing experiments and Cd exposure) (Figure 4.1A).

2.3. Induction of male daphnids and crossing experiments

In total 20 clonal lineages were used in this experiment. In a first generation for each clone, 5 juveniles (<24h) were put in 50 mL modified M4-medium (=P-generation= 1st generation figure 4.1A). These daphnids were feed daily at 2*10^5, 4*10^5 and 6*10^5 algae cells/ daphnid in their first, second and third week of their life respectively under a light:dark cycle of 8h:16h. The M4-medium of these organisms was renewed once a week. A second generation (=F1-generation) was established by using 5 juveniles (<24h) of the previous generation (<24h) from the third brood. Medium renewal, feeding and light: dark cycle was the same as the previous generation. If there were males in this second generation, they

were kept separately (= 3th generation figure 4.1.A.) to avoid intraclonal crossing. The induction of males lasted until all crossings were finished.

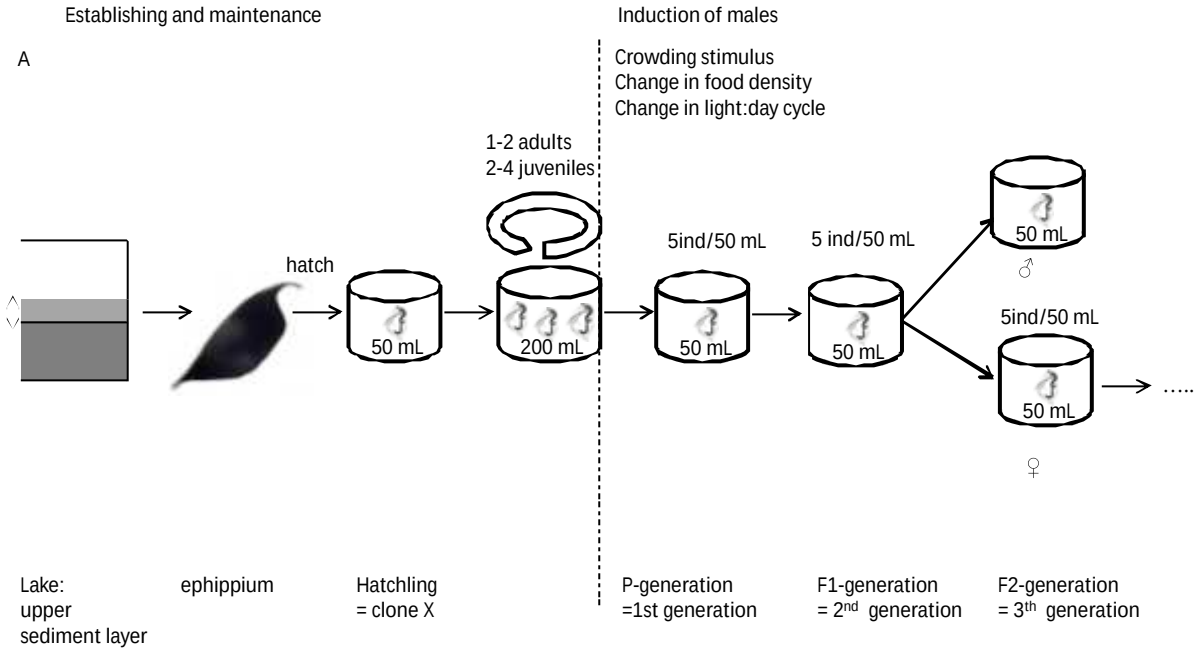

Figure 4.1A: Schematic overview of the design for the induction of male Daphnids. The design was followed for each of the clonal lineages originating from the field population.

Crossing was established by taking 5 females (= organisms who carried eggs) of one clone and put together with 5 males of another clone in 50 mL modified M4-medium (Figure 4.1.B). So in total 10 organisms were placed in 50 mL modified M4 medium. The crossings were checked every two days and asexual offspring were removed manually. The ephippia produced in the first week were removed and only ephippia from the second and third week were kept at 4°C in the dark in carbon filtered water. After at least two months, ephippia were manually decapsulated and placed in 50 mL cups filled with aerated carbon filtered tap water. Each ephippia was kept separately and only one hatched juvenile (=offspring clone) was used for further experiments. These hatchlings were given a clone name.

B

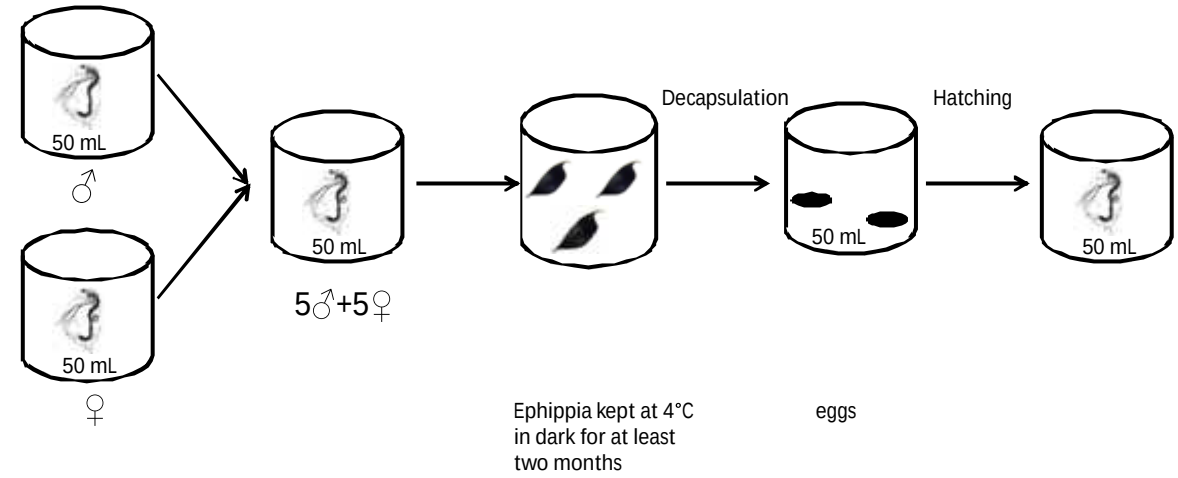

Figure 4.1.B: Schematic overview of the design for crossing experiment. Male (**>**) and female (**+**) daphnids are different clones, so intraclonal crossing was excluded.

2.4. Test design of Cd experiment

The test design of the Cd experiment is presented in Figure 4.2. The juveniles (<24h old) from a single clone (as well for parental as for offspring clones) were pooled and 3 juveniles were randomly chosen from this pool to start a new generation of this lineage. Hence, each clone was presented by 3 individual replicates maintained in polyethylene cups containing 50 mL of modified M4 medium. The juveniles of this generation were used to start a new generation at 20°C and a first generation at 24°C. For each clone, juveniles (<24h) produced by these three adults were pooled together and 8 juveniles (<24h) were randomly picked out to start the P-generation in each temperature (P-generation in Figure 4.2), so 4 juveniles were transferred to 24°C and 4 juveniles were kept at 20°C. Each juvenile was transferred individually to a separate 50 mL polyethylene beaker. The individuals in this P-generation then served as the mothers for producing the following generation. At the third or fourth brood, one juvenile (<24h) (F1) from one mother organism (P) was selected and were placed individually in 50 mL polyethylene vessel to establish the following generation

(F1-generation). One juvenile (F2) from the third or fourth brood from one organism (F1) was used to start the following generation (F2). At the third brood of this generation (F2), two juveniles (<24h) (F3) from each mother organisms (F2) were selected and placed individually in 50 mL polyethylene vessels with modified M4 medium, in a control treatment (no added Cd) and a Cd treatment of 5 µg Cd/L (added as $CdCl_2 \cdot H_2O$) and were subsequently monitored for 21 days following OECD test guideline No. 211 (OECD, 1998). Control exposures (no Cd added) and Cd exposures at 20°C and 24°C with all clones (parental and offspring clones) were run in parallel. Maternal effects can be ruled out in the estimation of genetic variance, as for each clone in each Cd concentration, each of the three replicate individuals (juveniles) being exposed originated from a different mother organism. Based on daily observations the following traits were determined: total reproduction at day 21 (R_0) and population growth rate (see Eq.2.3).

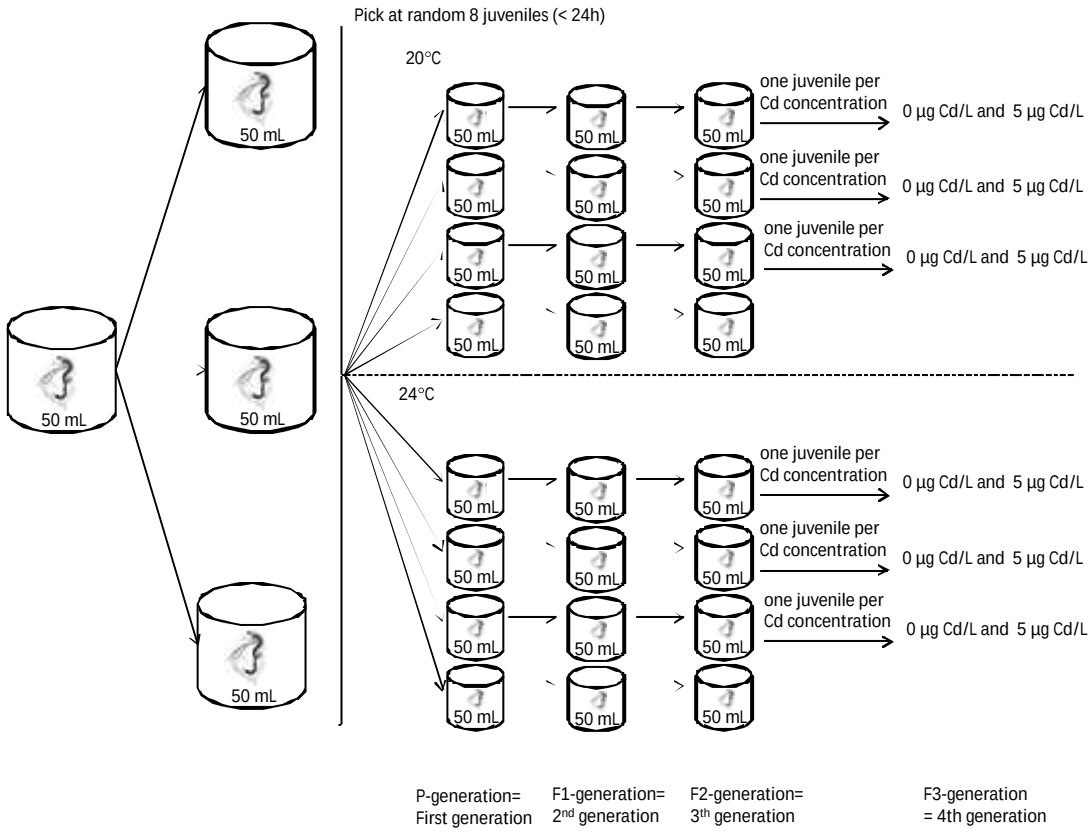

Figure 4.2: Schematic overview of the experimental design that was followed for ech clonal lineage.

Samples for analysis of dissolved Cd analysis were taken every week. pH, dissolved oxygen and temperature were measured at least twice a week and dissolved organic carbon (DOC) samples were taken at the beginning and end of the experiment.

2.5. Chemical analyses

The chemical analyses during the Cd experiment are described in Chapter 2 (§2.4)

2.6. Statistical analyses

As described in previous chapters (chapters 2 and 3), there are different ways to standardize the level of genetic variation of a (fitness) trait. The additive genetic variance (V_A) can be presented as a proportion of the total phenotypic variance (V_P), and this ratio is called the (narrow sense) heritability (h^2) (Lynch and Walsh, 1998):

$$h^2 = V_A / V_P \qquad \text{(Eq. 4.1)}$$

$$V_P = V_A + V_D + V_E \qquad \text{(Eq. 4.2)}$$

$$V_G = V_A + V_D \qquad \text{(Eq. 4.3)}$$

$$H^2 = V_G / V_P \qquad \text{(Eq. 4.4)}$$

$$CV_G\,(\%) = 100 * \sqrt{V_G} / \mu \qquad \text{(Eq. 4.5)}$$

$$CV_A\,(\%) = 100 * \sqrt{V_A} / \mu \qquad \text{(Eq. 4.6)}$$

Where V_P = total phenotypic variance, V_G = total genetic variance, H^2 = broad-sense heritability, h^2 = narrow-sense heritability, CV_G = the (total) genetic coefficient of variation,

and CV_A = the additive genetic coefficient of variation (Lynch and Walsh 1998; Klerks et al. 2011; Falconer and Mackay 1996).

The Restricted maximum likelihood (REML) method was used to estimate the variance components of additive genetic effects (σ^2_A), dominance effects (σ^2_D) and residual effects (σ^2_E) and this was based on the Animal Model (see Supplementary Material S4.1 for detailed information). The covariance matrix was calculated using the Banded Toeplitz Method. Calculations were performed using SAS 9.2. (SAS Institute Inc, Cary, NC, USA). The model can be expressed in matrix form (linear mixed model):

$$Y = Xb + Z_1\mu_1 + Z_2\mu_2 + e \quad \text{(Eq. 4.7)}$$

Where Y=vector of trait values of all tested individuals

b=vector of fixed effect (population mean)

μ_1 = vector of additive genetic effects (random effect)

μ_2 = vector of dominance genetic effects (random effect)

e= vector of residual effects (random effect)

X = unit vector

Z_1 and Z_2 = matrices of random effects (see annex for more details)

Based on the estimated values of the three variance components and the variance-covariance matrix of these estimates, the following parameters and their variance were calculated, based on Lynch and Walsch (1998): narrow sense heritability (h^2), additive genetic coefficient of variation (CV_A), broad sense heritability (H^2), genetic coefficient of variation (CV_G) (see Supplementary Material, S4.2. for detailed information). Significance of differences between estimates of the parameters in the control and the Cd treatment was assessed by calculating z scores:

$$z = (\mu_{Cd} - \mu_{Control}) / \sqrt{(s^2_{Cd} + s^2_{Control})} \quad \text{(Eq.4.8)}$$

Where μ= estimates and s= standard errors. In the case of traits being tested against a value of zero, the formula is reduced to the ratio between the estimate and its standard error. The null-hypothesis states that the means of two treatments are equal. If $|z| > 1.96$, the means are considered significantly different, and the null-hypothesis is rejected ($p<0.05$).

3. Results

3.1. Chemical analyses

The physico-chemistry of the test media is presented as supportive information (Table S4.1). DOC ranged between 4.6 and 5.6 mg/L and pH between 7.5 and 7.6. The mean dissolved Cd concentrations (mean of old and new medium) differed at most 27% from the nominal Cd concentration. The Cd concentration in the old medium was on average 36% lower than in the new medium.

3.2. Population means

Values of traits for all individuals, all clones and all environments are given in Supplementary material, Table S4.2 – Table S4.48. Population means of total reproduction (R_0) and population growth rate (r_m) are reported in Table 4.1. At 20°C, *D. magna* exposed to cadmium exhibited, compared to the control, a significantly lower reproduction during 21 days (-23%) and significantly lower population growth rate (-9%). Cd had a more pronounced effect at 24°C on total reproduction (-88%) and population growth rate (-60%).

Table 4.1: Estimated population means of the fitness traits ± standard error. An asterisk (*) indicates a significant ($p<0.05$) difference between control group and Cd treatment in one temperature treatment (20°C and 24°C). Numbers between parentheses indicates the % difference between the Cd and the control treatment.

	20°C	20°C + Cd	24°C	24°C + Cd
Total reproduction (R_0)	105.55 ± 4.70	81.23 ± 3.92* (-23%)	98.13 ± 5.10	11.69 ± 2.83* (-88%)
Population growth rate (r_m)	0.38 ± 0.01	0.35 ± 0.01* (- 9%)	0.40 ± 0.02	0.16 ± 0.02* (- 60%)

3.3. Narrow sense heritability, additive genetic coefficient of variation (CV_A), additive genetic variance and dominance genetic variance

The estimates of the narrow sense heritability, additive genetic coefficient of variation of total reproduction (R_0) and population growth rate (r_m) are presented in Figure 4.3. The estimates of additive and non-additive components of variance of total reproduction (R_0) are presented in Table 4.2. The estimates of additive and dominance components of variation of population growth rate (r_m) could not be estimated in 20°C because of lack of data and too low variation between traits. Variance components at 24°C are presented in Table 4.3. Additive and dominance variance was not significantly greater than 0 for r_m at 24°C. Covariance estimates are presented in Supplementary Material (Table S4.49 – Table S4.54).

Table 4.2: Estimates of additive variance, dominance variance and error variance in natural *Daphnia magna* population for total reproduction (R_0). Values represent estimates ± standard error. An asterisk indicates that the estimate is significantly > 0.

	20°C		24°C	
Genetic components	0 µg Cd/L	5 µg Cd/L	0 µg Cd/L	5 µg Cd/L
Additive variance (V_A)	91.33 ± 191.01	93.64 ± 169.99	127.97 ± 276.5	104.06 ± 50.89*
Dominance variance (V_D)	1604.65 ± 572.85*	554.52 ± 408.6	1042.78 ± 594.72*	169.99 ± 108.27
Genetic variance (V_G)	1696.0 ± 551.3*	648.2 ± 386.2*	1170.8 ± 612.8*	274.1 ± 96.8*
Error variance (V_E)	920.71 ± 137.72*	884.1 ± 143.51*	1830.75 ± 263.36*	198.86 ± 30.14*
Phenotypic variance (V_P)	2616.7± 524.9*	1532.2 ± 330.9*	3001.5 ± 548.3*	472.9 ± 86.4*

Table 4.3: Estimates of additive variance, dominance variance and error variance in natural *Daphnia magna* population for population growth rate (r_m). Values represent estimates ± standard error. An asterisk indicates that the estimate is significantly > 0.

	24°C	
Genetic components	0 µg Cd/L	5 µg Cd/L
Additive variance (V_A)	0.003 ± 0.003	0.001 ± 0.002
Dominance variance (V_D)	0.007 ± 0.007	0.012 ± 0.006
Genetic variance (V_G)	0.01 ± 0.01	0.013 ± 0.01
Error variance (V_E)	0.012 ± 0.002*	0.013 ± 0.001*
Phenotypic variance (V_P)	0.022 ± 0.01*	0.026 ± 0.01*

For total reproduction (R_0) (Table 4.2), an additive genetic variance significantly >0 was detected in 24°C + Cd but not in the other two treatments. A significant dominance variance (> 0) was only found in the two control treatments but not in the Cd treatments. For R_0, h^2 (Figure 4.3) ranged between 0.04 to 0.2, but was only at 24°C + Cd significantly greater than 0. A significant Cd effect on h^2 was found at 24°C (compared to the control). The additive coefficient of variation (CV_A) ranged between 4.11% and 86.98%. CV_A was significantly > 0 in 24°C + Cd. At 24°C there was a significant Cd effect.

For population growth rate (r_m) (Table 4.3), no significant additive and dominance components were found in the control and Cd treatment. The h^2 ranged between 0.04 and 0.13. No effect of Cd was found on h^2 at 24°C. Additive genetic coefficient of variation (CV_A) was approximately the same in the two treatments (11.3% and 11.7%) and no Cd effect was found either (compared to the control) (Figure 4.3).

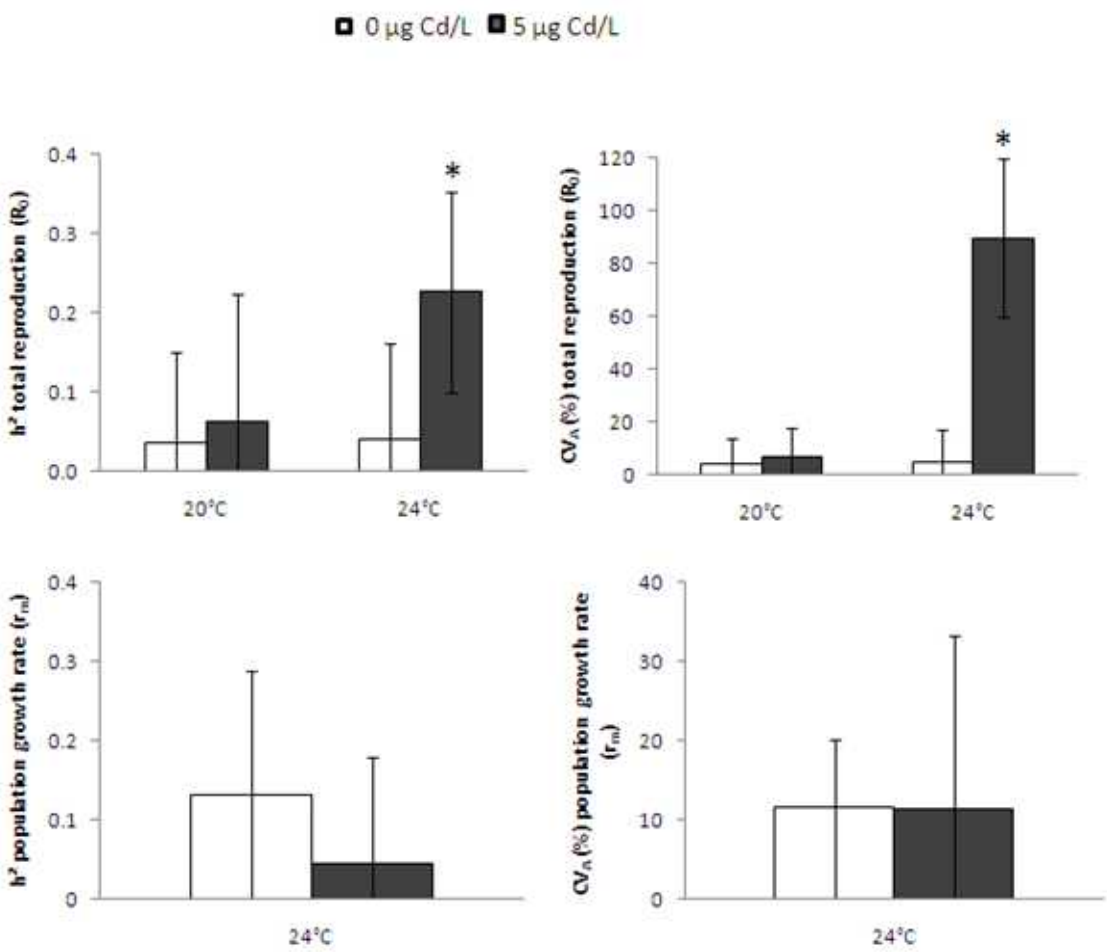

Figure 4.3: Narrow sense heritability (h^2) and additive genetic coefficient of variation (CV_A) for different fitness traits in a *D. magna* population. Error bars represent the standard deviation. An asterisk (*) indicates a significant difference between the Cd and the control treatment within a temperature treatment ($p<0.05$).

3.4. Broad sense heritability and genetic coefficient of variation (CV_G).

Broad sense heritability (H^2) (Figure 4.4) ranged between 0.37 and 0.69 and was significantly > 0 in all treatments for total reproduction (R_0). There was no Cd effect on H^2 in

either of the two temperature treatments. Genetic coefficient of variation (CV_G) for total reproduction ranged between 30.02% and 146.91%, and there was a Cd effect at 24°C.

Broad sense heritability (Figure 4.4) and genetic coefficient of variation could not be estimated in 20°C for population growth rate (r_m). H^2 ranged between 0.41 and 0.49 and CV_G between 24% and 73% for CV_G at 24°C. There was no Cd effect on H^2. A significant Cd effect was found for CV_G.

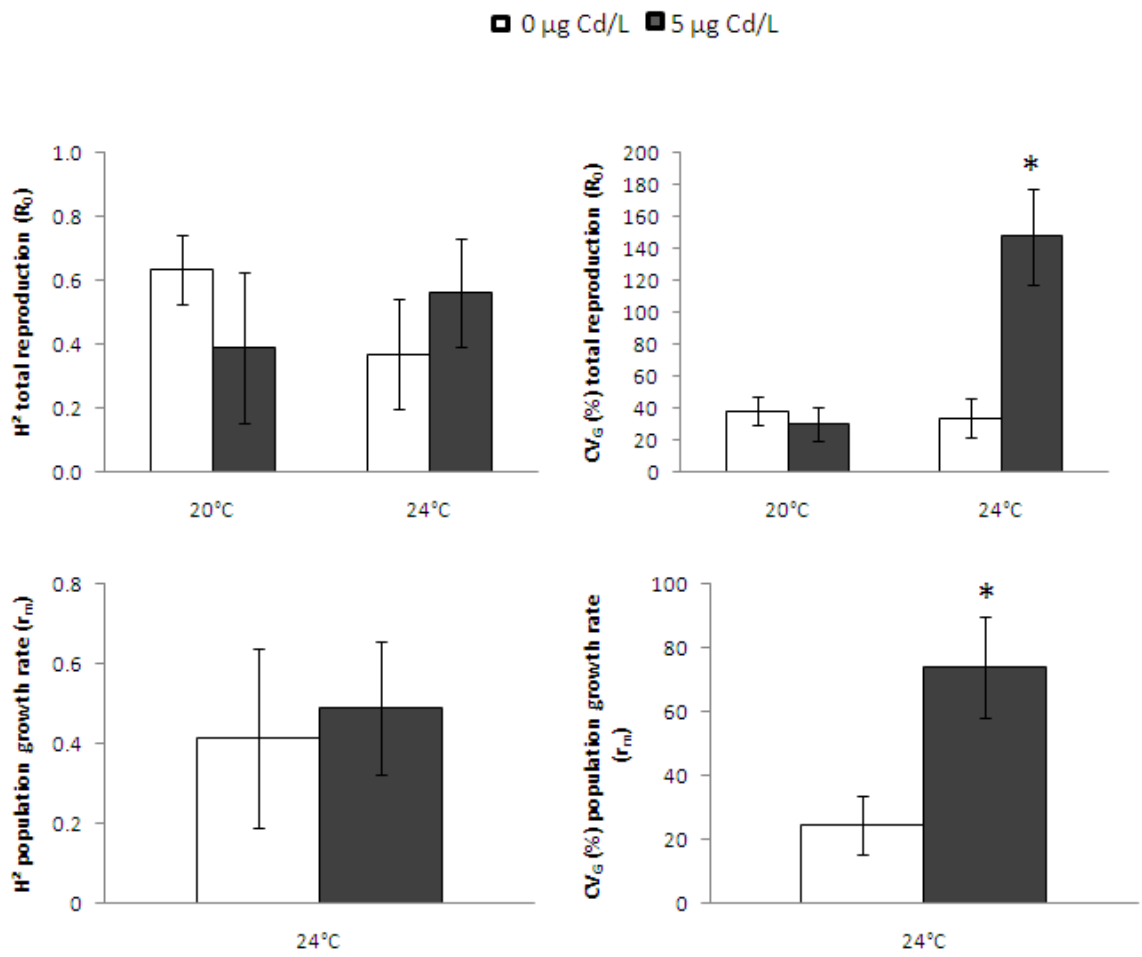

Figure 4.4: Broad sense heritability (H^2) and genetic coefficient of variation (CV_G) for different fitness traits in a *D. magna* population. Error bars represent standard deviation. An asterisk (*) indicates a significant difference between the Cd and the control treatment within a temperature treatment ($p<0.05$).

4. Discussion

This study indicates the existence of genetically determined differences of fitness traits within a *Daphnia magna* population under temperature and Cd stress. In all

treatments, a CV_G and $H^2 > 0$ was detected for both fitness traits. For total reproduction (R_0) similar results were found as in Messiaen et al., 2010 (chapter 3): there was no Cd effect on H^2 at both temperatures and there was a significant Cd effect on CV_G at 24°C (compared to a control). Similarly, at 24°C, an effect of Cd was found on the CV_G of r_m, but not on the H^2 of r_m. All this indicates that with increased temperature, there may be an increased micro-evolutionary potential under Cd stress. Under long-term multigenerational exposure this may lead to stronger shifts in genotype frequencies due to Cd at higher temperature. Compared to Messiaen et al. (2010) (chapter 3), the adverse effect of Cd at higher temperature was more pronounced. The Cd-induced stress at 24°C was clearly higher, both based on total reproduction (R_0) (i.e. -92% in this chapter versus -20% in chapter 3) and on population growth rate (r_m) (-60% in this chapter versus -7% in chapter 3). As the CV_G is also partly determined by the population mean of the fitness trait, the difference in population mean explains the observed difference between both studies, as the genetic variance (V_G) was only a factor 1.3 different between this chapter and chapter 3 at 24°C and Cd. In summary, based on the result of this study and the previous chapter, we can see that following a clonal approach, there is a micro-evolutionary potential (expressed as CV_G) expected at higher temperature under sublethal Cd stress of (nominal) 5 µg Cd/L (compared to the control).

However, the total genetic variability detected does not necessarily equate to inheritance. Additive genetic variance (i.e. significantly different from 0) was detected in the 24°C + Cd treatment for total reproduction (R_0), but not in other treatments. The observed total genetic variance in the three other treatments largely consists of large non-additive variance (i.e. dominance variance) (Table 4.2). A low (and not significant) narrow sense

heritability was also detected for those three treatments, and a significant h^2 (equal to 0.22) was only observed in the 24°C + Cd treatment. Several studies with *Drosophila* populations have reported an increase in heritable variation in response to stressed conditions (Hoffmann and Hercus, 2000). Klerks and Moreau (2001) found similar (non significant) values of h^2 (range between 0 and 0.2) in a sheephead minnow population (*Cyprinodon variegatus*) exposed to Zn, phenanthrene and mixtures of Zn, phenanthrene, Ni, Barium and three polycyclic aromatic hydrocarbons. The higher h^2 at 24°C + Cd can be explained by lower dominance and residual variances in comparison with the other treatments. The amount of additive variance was similar between all treatments. Sgrò and Hoffmann (1998) indicated an increased h^2 of fecundity in a *Drosophila* population under stress (combination of ethanol, cold shock and low nutrition), reflecting an increase in the additive genetic variance. They found no differences in residual variance across the treatments. The finding of a significant additive genetic variance of fitness at 24°C + Cd is in contrast with the results of Chaumot et al. (2009), who did not detect a significant additive genetic component of survival time in a *Gammarus* population under lethal Cd stress of 20 µg Cd/L. The latter finding made Chaumot et al. (2009) to postulate that observed exceptional cases of adaptation of field populations would be permitted only by fixation of rare alleles. Our study however indicates that adaptation to Cd stress in a *D. magna* population can occur through standing genetic variation.

5. Conclusion

The observed genetic variability in tolerance to contaminants in several *Daphnia magna* populations does not necessarily indicate an adaptive potential across sexual generations. Our results indicate that there is a significant additive genetic variance for total

reproduction in the 24°C + Cd treatment, which indicates that this higher fitness may be inherited by the following generations. It also suggests that the fitness under stressed conditions may increase over sexual generations due to natural selection. Yet, this result should be interpreted with caution as for population growth rate r_m, usually considered a better prediction of fitness in the field, a significant additive genetic variance was not observed. Discrepancies between different studies and species indicate that more research is needed to determine the potential for adaptation to contaminants in natural populations.

Chapter 5: Between and within population variability in *Daphnia magna* populations exposed to Cd stress.

Abstract- This study examines the variability of fitness traits between and within 11 natural *Daphnia magna* population exposed to Cd stress. To this end, a set of hypotheses related to micro-evolutionary responses were tested. Life-table experiments with a control and Cd treatment (5 µg Cd/L) were conducted with 12 *D. magna* clones originating from 11 *Daphnia magna* populations collected from 11 (Cd) unpolluted lakes. Several fitness traits were monitored during 21 days: total reproduction (R_0), population growth rate (r_m), reproduction at first brood and maturation rate. Our results indicate a 3-fold difference in Cd tolerance of total reproduction (R_0) between the most sensitive and the most tolerant population. A significant population effect was found for Cd tolerance of population mean of total reproduction (R_0), population growth rate (r_m) and maturation rate. Not all populations exhibited a significant micro-evolutionary potential (expressed as CV_G and H^2) under control and Cd exposure. Under Cd stress, 45% and 27% of the populations had a significantly higher CV_G or H^2, respectively, compared to the control for total reproduction (R_0). This increase of CV_G/H^2 may increase the micro-evolutionary potential under Cd stress (compared to the control) and may eventually lead to a stronger (but different between populations) reduction of clonal diversity (compared to the control) in a natural setting. Overall, our results suggest that there is within genetic variability in *Daphnia magna* populations, indicating that populations originating from other habitats may have a different micro-evolutionary potential under Cd stress.

1. Introduction

Natural populations can consist of a large number of genetically different individuals, upon which natural selection can act. Although the use of monoclonal laboratory populations may be useful for standardization of laboratory procedures (Baird, 1992), toxicity tests with these may be of little relevance for predicting the capability of natural populations to adapt to a changing environment (Forbes and Depledge, 1996). Furthermore, natural populations are exposed to various natural and anthropogenic stressors, and local adaptation leads to a higher average fitness of the resident population in the local habitat compared to genotypes from other habitats (Kawecki and Ebert, 2004). This local adaptation has been widely reported for *Daphnid* populations with regard to predation pressure (Cousyn et al., 2001, Boersma et al., 1999), land-use (Coors et al., 2009) and metal contamination (Morgan et al., 2007; Lopes et al., 2005; Lopes et al., 2006).

The aim of the above-cited studies was mainly to compare the tolerance for certain stressors between populations originating from a stressed-environment (polluted) and populations originating from a non-stressed environment (non-polluted). Only a few studies have compared the effect of a pollutant among populations, living in habitats with no severe contamination with the pollutant of interest. Barata et al. (2002c) reported no difference in EC_{10} of Cd and EC_{10} and EC_{50} of λ-cyhalothrin among *D. magna* populations originating from three different pristine habitats. A study with four *D. magna* populations originating from four pristine habitats showed significant among-population differences of neonate longevity responses at 10 µg Cd/L but no effect on fitness (i.e. $e^{clutchsize/time\ to\ first\ reproduction}$) at sublethal Cd concentrations (0-2 µg Cd/L) (Barata et al., 2002b).

As indicated in the previous chapters, the potential of genetic adaptation of populations to chemicals (or at least the potential for such adaptation) can be determined by examining the genetic variation and/or heritability of fitness traits. Barata et al. (2002a) for example, found significant genetic variation for cadmium tolerance within natural populations, suggesting a potential to acquire resistance to Cd stress. Messiaen et al. (2010) found a significant genetic coefficient of variation in a *D. magna* population under temperature and Cd stress. Barata et al. (2000b) also found significant genetic variability for reproduction and time to first brood under Cd and ethylparathion exposure within a *D. magna* field population. Chaumot et al. (2009) demonstrated genetic variability in acute Cd sensitivity within a *G. fossarum* population although the additive genetic variability was negligible. To our knowledge, only a few studies have investigated the difference in micro-evolutionary potential between multiple populations. Barata et al. (2002b) showed that (1) there was significant heritability (i.e. significant differences in lethal tolerance among clones) in two populations for longevity responses of neonates exposed to 10 µg Cd/L and (2) the heritability levels for this lethal tolerance was similar for those two populations. In a study of Agra et al. (2010), two *D. longispina* populations, one originating from a habitat impacted by acid mine drainage and one from an unimpacted habitat, had similar (high) levels of (broad sense) heritability in tolerance to Cu and Zn.

The aim of the present study was to address some micro-evolutionary aspects by testing a set of hypotheses related to the micro-evolutionary potential of 11 natural populations of *Daphnia magna* exposed to a control and a sublethal cadmium concentration. The first hypothesis - as proposed by Barata et al. (2000b) - is that a population exposed to cadmium will exhibit more genetic variation for life-history traits than the control

population. As with increasing Cd concentration, there will be a lower fitness, and following Hoffmann and Hercus (2000), this will in turn result in a higher genetic variability of life-history traits in a high cadmium environment compared to the control (hypothesis 1). Also, we hypothesize that there will be a difference between population means (hypothesis 2) as well as a difference of the within-population genetic variability (expressed as H^2 or CV_G) between those 11 populations (hypothesis 3) originating from different habitats with no severe Cd contamination. We searched for a broad range of ponds containing the keystone species *Daphnia magna* and differing in 3 important selection factors for cladoceran communities: (1) fish presence/ absence, (2) low/high parasite prevalence and (3) low/high intensity of agricultural land use (Rousseaux et al., in prep.). We conducted 21-day life-table experiments in a control (0 µg Cd/L) and 5 µg Cd/L. All experiments were conducted with eleven populations consisting of 12 different *D. magna* clones hatched from different ephippia, which had previously been collected from eleven unpolluted Cd ponds. By measuring different life-history traits of individuals of each genotype kept under two different environmental conditions (control and 5 µg Cd/L), estimates of Cd tolerance were obtained. So, fitness in two exposure (Control and Cd exposures) was assessed, as the response of fitness in a Cd treatment towards the control (Cd tolerance). The genetic coefficient of variation and broad sense heritability were determined as measures of the micro-evolutionary potential of the population (Barata et al., 2002b; Lynch and Walsch, 1998). We also investigated which habitat characteristics may explain differences in population means and quantitative genetic variability among *D. magna* populations.

2. Material and methods

2.1. Sampling of *Daphnia magna* populations

Samples of the recent dormant egg bank of 11 ponds were sampled in January-March 2007. In winter the resting stages of water fleas accumulate in the sediment and by sampling the upper 2 centimeters of sediment using a sediment corer, we ensured having the recent zooplankton community. Eight ponds were located in Flemish-Brabant (Leuven) and three ponds were located in Western Flanders (Knokke). We selected the ponds in cooperation with the laboratory of Aquatic Ecology and Evolutionary Biology (KULeuven) who obtained information on several Belgian aquatic systems through earlier studies: 126 farmland ponds (Declerck et al. 2006); 34 shallow lakes (Declerck et al. 2005); 32 shallow lakes in nature reserve 'De Maten' (Michels et al., 2001) and approximately 20 shallow lakes and ponds in the region of Leuven. We searched for a broad range of ponds containing the keystone species *Daphnia magna* and differing in 3 important selection factors for cladoceran communities: (1) fish presence/ absence, (2) low/high parasite prevalence and (3) low/high intensity of agricultural land use (Rousseaux et al., in prep.). A overvieuw is presented in Table 5.1. 8 ponds are located in Flemish-Brabant (Belgium) with MO in Moorsel, which is a concrete storm water basin with occasional inflow of both agricultural field run-off and waste water overflow. Ponds TER1 and TER2 are located close to Neerijse (farm Tersaert) but are not inter-connected. Those three ponds are fish-less and have an impact of land-use. The fourth pond (LRV) is located in Langerode. OHZ and ZW4 are both located in Oud-Heverlee, whereas pond OM2 and OM3 are in Heverlee (Oude Meren, Abdij van het Park). With exception of OM2 and OM3 the ponds are not interconnected. All of the ponds provide a permanent habitat for *Daphnia magna*. Ponds KNO15, KNO17 and KNO52 are located close

to Knokke. Detailed information on the habitatcharacteristics are presented in Supplementary Material Table S5.90 - Table S5.93.

Table 5.1: Overview of the selection factors absent or present in the different ponds. The three selection factors are presented by + (presence) or – (absence). More detailed information on the ponds are given in Supplementary Material Table S 5.90- Table S5.93.

Pond	Location	Selection factors		
		Fish-presence	Parasite-presence	Land-use intensity
KNO15	Knokke	-	+	-
KNO17	Knokke	+	+	+
KNO52	Knokke	-	-	-
LRV	Langerode	+	-	-
MO	Moortsel	-	+	+
OHZ	Oud-Heverlee	+	+	-
OM2	Heverlee	+	+	+
OM3	Heverlee	+	-	+
TER1	Neerijse	-	-	+
TER2	Neerijse	-	-	-
ZW4	Heverlee	+	-	-

2.2. Maintenance and culturing of *D. magna* clones

Sediment samples containing cladoceran resting egg banks were stored at 4°C until the start of incubation. Upon hatching, a single hatchling from each ephippium was selected to establish a clonal lineage (Ebert et al., 1993). Ephippial eggs of *D. magna* are produced by sexual reproduction, so each clonal lineage can be considered genetically distinct (Barata et al., 2000b). The dormant eggs were isolated by means of 'sugar flotation method' (Onbe, 1978; Mareus, 1990), by transferring filtered sediment with oversaturated sugar solution

(1000 g sugar in 1000 mL distilled water) to Falcon tubes that were centrifugated and decanted for two cycli (3 minutes and 10 minutes at 3000 rpm). The remaining sediment was checked visually and any remaining dormant eggs were picked out manually. All isolated eggs put in ADaM medium (Aachener Daphnien Medium, Klüttgen et al., 1994) in a climate room at 20°C and in a 16:8 light: dark cycle photoregime. Medium was refreshed every 8 to 9 days. Hatchlings were isolated daily. The hatchlings were further cultured as clonal lineages in 300 mL vessels with aged tap water and were fed two times a week with $100*10^6$ cells of *Scenedesmus obliquus*. At December 2008, 12 randomly selected clones from each population were transported to the lab of aquatic ecology and environmental toxicology (UGent). The maintenance of all the clones with field populations in this lab were performed under standardized laboratory conditions, i.e.: 20°C, a light:dark cycle of 16h:8h and in modified M4-medium (see chapter 2, §2.1). Each clone was kept in 50 mL polyethylene vessels. The culture medium was renewed once a week. With every medium renewal, the next generation of each clone was established by randomly picking 1 to 2 juveniles and/or 1 or 2 adult daphnids (daphnids which carried eggs) of the previous generation. Thus, with every renewal, 1 to 4 daphnids were placed in 50 mL polyethylene vessels, and the next generations of each clone were fed approximately 0.5 mg dry wt per day per 50 mL.

2.3. Test design

Based on Messiaen et al. (2010) a 5 µg Cd/L was selected as the sub-lethal Cd concentration. In a first step, one juvenile (<24h) of the third of fourth brood of the previous generation of each clone of each population was put individually in a 50 mL vessel with modified M4 medium (=P-generation). To eliminate maternal effects in the test generation, a second generation (=F1-generation, Figure 5.1) of each clone was established. So, all the

juveniles (<24h) of the P-generation of each clone were pooled, and each juvenile of each clone was put individually in a 50 mL polyethylene vessel filled with modified M4-medium, so each clone consisted in total of four individually kept juveniles (=F1-generation). This generation was followed during 21 days (OECD, 1998). The organisms were fed daily with a 3:1 mixture (based on cell numbers) of the algae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* equivalent to 250 µg dry wt/*Daphnia*, 500 µg dry wt/*Daphnia* and 750 µg dry wt/*Daphnia* in the first, second and third week of their life, respectively. The medium was renewed three times a week.

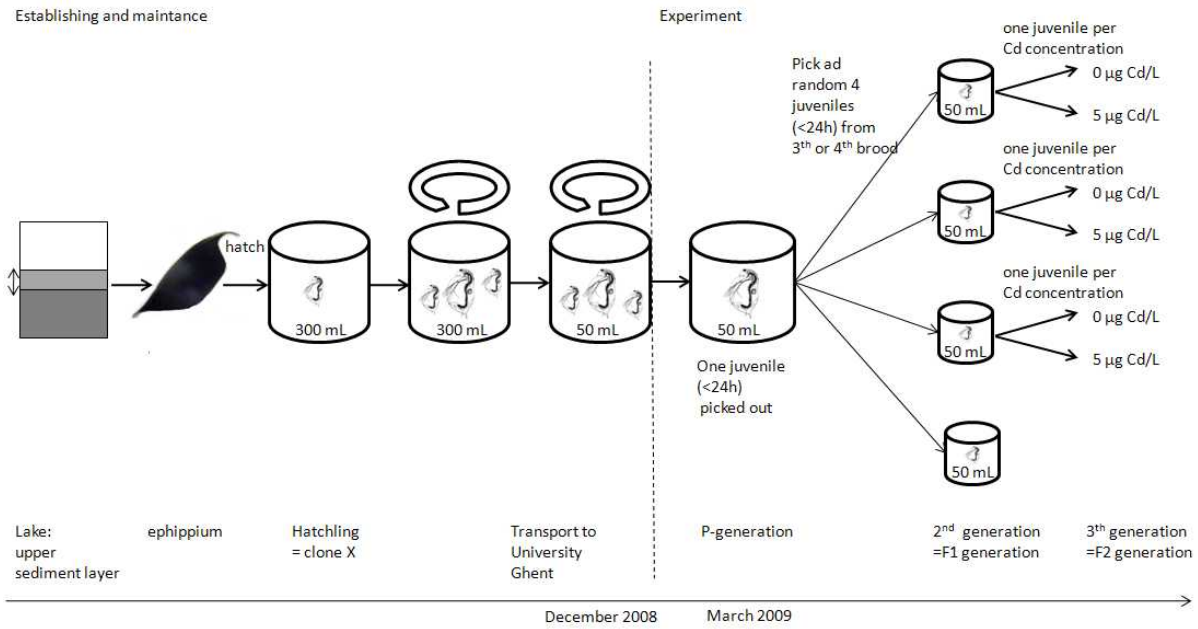

Figure 5.1: Schematic overview of experiment design for one clone originating from one field population. The same design was followed for all clones.

Cd exposures of all clones were simultaneously initiated with juveniles (<24h old) (=F2 generation) from the third or fourth brood of the previously generation (=F1 generation). Three of the four Daphnids (=F2 generation) of each clone were randomly selected to start the new generation. One juvenile (<24h) of each *Daphnia* was placed individually in a 50 mL polyethylene vessel with modified M4 medium (control) and one juvenile (<24h) of each *Daphnia* in modified M4 medium with 5 µg Cd/L (added as $CdCl_2 \cdot H_2O$)

and they were subsequently monitored for 21 days following (OECD, 1998). As such, maternal effects can be ruled out in the estimation of genetic variance, as per Cd concentration, each clone consisted of 3 replicates originating from a different mother organism, thus maternal variance is 100% included in residual variance (Lynch and Walsh, 1998). All Cd exposures with all clones were simultaneously initiated, resulting in a precise estimate of population responses. The organisms were fed daily as described above, and the test medium was renewed three times a week.

Based on daily observations the following traits were determined: survival, maturation rate, reproduction (number of juveniles) at first brood and total reproduction at day 21 (R_0). Population growth rate (r_m) was calculated according to Euler-Lotka equation (Lotka, 1913) (see Eq 2.3, Chapter 2).

As maternal effects were taken into account, Cd tolerance of each trait was determined as the ratio of the value of the fitness trait in the control and the value of the trait in the Cd exposure treatment.

2.4. Sampling and determination of habitat characteristics of 11 ponds

In April 2009 sediment and water samples were taken to determine metal concentrations. The upper layer (approximately 10 cm) of the sediment was taken to determine Ni, Cu, Pb, Zn and Cd. Sediment destruction for total metal content was done by acid microwave digestion. Ni, Cd, Cu, Zn and Pb were analyzed using flame AAS (Spectra AA 100-Varian) and/or a graphite furnace AAS (Zeeman, Spectra AA300-Varian).

To determine Cu, Ni, Pb, Cd, Na, Ca and Mg concentrations in the water, AAS tubes and Falcon tubes were put >24h in advance before sampling in a 0.1%vv HNO_3 bath. A day

before sampling, the tubes and filters were rinsed three times with 0.1% HNO_3 and three times with Ultra-Pure Water (Chemlab, Zwevezele, Belgium). At the sampling place, the tubes were also rinsed three times with the respective lake water before sampling. At each location three times 50 mL samples were put in Falcon tubes. To determine the metal concentrations, the samples were centrifugated for 15 minutes at 2000 rpm in the lab (Centra 8, Thermolife Sciences, Belgolab). For each lake, three times 10 mL samples were non-filtered (for the measurement of total metal concentration). The concentrations of Cu, Ni, Pb, Cd, Ca and Mg were measured with ICP-MS (inductive coupled plasma mass spectrometry, Perkin-Elmer Elan DRC-e, Wellesley, MA, USA).

Fish-abundance was scored and parasite prevalence (%) of *Vorticella*, *Amoebidium, Binucleata* and *Pasteuria* was also determined. Fish abundance was categorised into five categories. The categories were: 0 = fish absent; 1 = only threespine stickleback *Gasterosteus aculeatus*, with less than 100 individuals caught during 5 minutes electrofishing; 2 = only threespine stickleback, with >100 individuals caught during 5 minutes electrofishing; 3 = diverse fish community including planktivorous fish such as *Rutilus rutilus*, *Gasterosteus aculeatus* and *Scardinius erythrophthalmus* at moderate densities; 4 = diverse fish community with the same planktivorous fish species at high densities. Land use intensity, a regional variable, was assessed by quantifying land use in the direct neighborhood (zone < 100 m) of the pond. In addition, the distance to the nearest crop field was also measured using satellite pictures (Google Earth; images dating from the spring after sampling). The percentage of arable land in a 200 m radius around each pond was quantified applying the GIS software package ArcView GIS 3.2a (ESRI, Inc.) to analyze topographical raster maps of the National Geographic Institute (1978–1993; scale: 1/10,000) and the land use coverage

database of Flanders (2001; resolution: 15 m). Those analyses were done by the laboratory of Aquatic Ecology and Evolutionary Biology (KULeuven).

2.5. Data treatment and statistical analyses

The existence of a population effect of different fitness traits in control, Cd treatment and for Cd tolerance (=ratio between fitness observed in Cd treatement and fitness observed in control treatment), was assessed with a Generalized Linear Model, with clone as a random factor nested in population as a fixed factor. The likelihood ratio Chi-square test was used to estimate the significance of population and clone(population) effects. In a first step fitness traits and values of Cd tolerance were log(x+1) transformed. Analyses were performed using SPSS (Statistics 17.0.1., IBM, NY).

To determine population means, CV_G, H^2, and their 95% confidence intervals, statistical analyses were performed as described in Chapter 2 (§2.4.). Post-hoc significant differences among populations for population mean, CV_G and H^2 are determined where more than 95% of the calculations yielded CV_G (population X) > CV_G (population Y) or H^2 (population X) > H^2 (population Y), population mean (population X) > population mean (population Y) (i.e. equivalent to a one-sided test at the 0.05 significance level). All calculations were performed in MATLAB 7.5.0.342 (Mathworks Inc) software.

Correlation between habitat characteristics and population parameters were performed using a nonparametric Spearman-rank correlation in Statistica 7.0 (Statsoft, Tulsa, OK) ($p<0.05$). To determine the metal ranking in sediment and water, in a first step for each of a metal concentration in the water/sediment, the populations were ranked. Afterwards, the ranking was summed for each of the populations, leading to a final ranking of metal

concentrations in the water and sediment. To determine correlations between metal ranking in water/sediment and the different fitness traits, each fitness trait was in a first step also ranked.

3. Results

3.1. Physico-chemical measurements during the test

The results of the chemical analyses during the exposures are summarized in Table S5.1 (Supplementary Material). DOC concentrations during the test ranged from 4.0 to 7.6 mg C/L. These concentrations were lower in the new medium than in the old. The pH ranged between 7.6 and 7.8. The mean Cd concentrations (mean of old and new medium) differ at most 13% from the nominal Cd concentration. The Cd concentration in the old medium was lower than in the new medium by average of 8%.

3.2. Population means and clone means

Values of all traits for all individuals, all clones and all populations are presented in Supplementary Material (Table S5.2 - S5.89). Population means for the different traits and populations are presented in Figure 5.2 – Figure 5.5.

The population mean of total reproduction (R_0) (Figure 5.2A) ranged between 37.4 juveniles per organism and 115.3 juveniles per organism. A significant Cd effect was found, although not for all populations (Figure 5.3A) (post-hoc analysis by nonparametric bootstrap resampling). Generalized Linear Model analysis indicates a population effect in control and Cd treatment (Table S5.106 - Table S5.107).

The population mean of population growth rate (r_m) (Figure 5.2B) ranged between 0.18 and 0.39. There was no Cd effect found for LRV, TER1, MO and KNO52 but there was an effect in all other populations. A population effect was found in control and Cd treatment (Table 5.108 - Table 5.109).

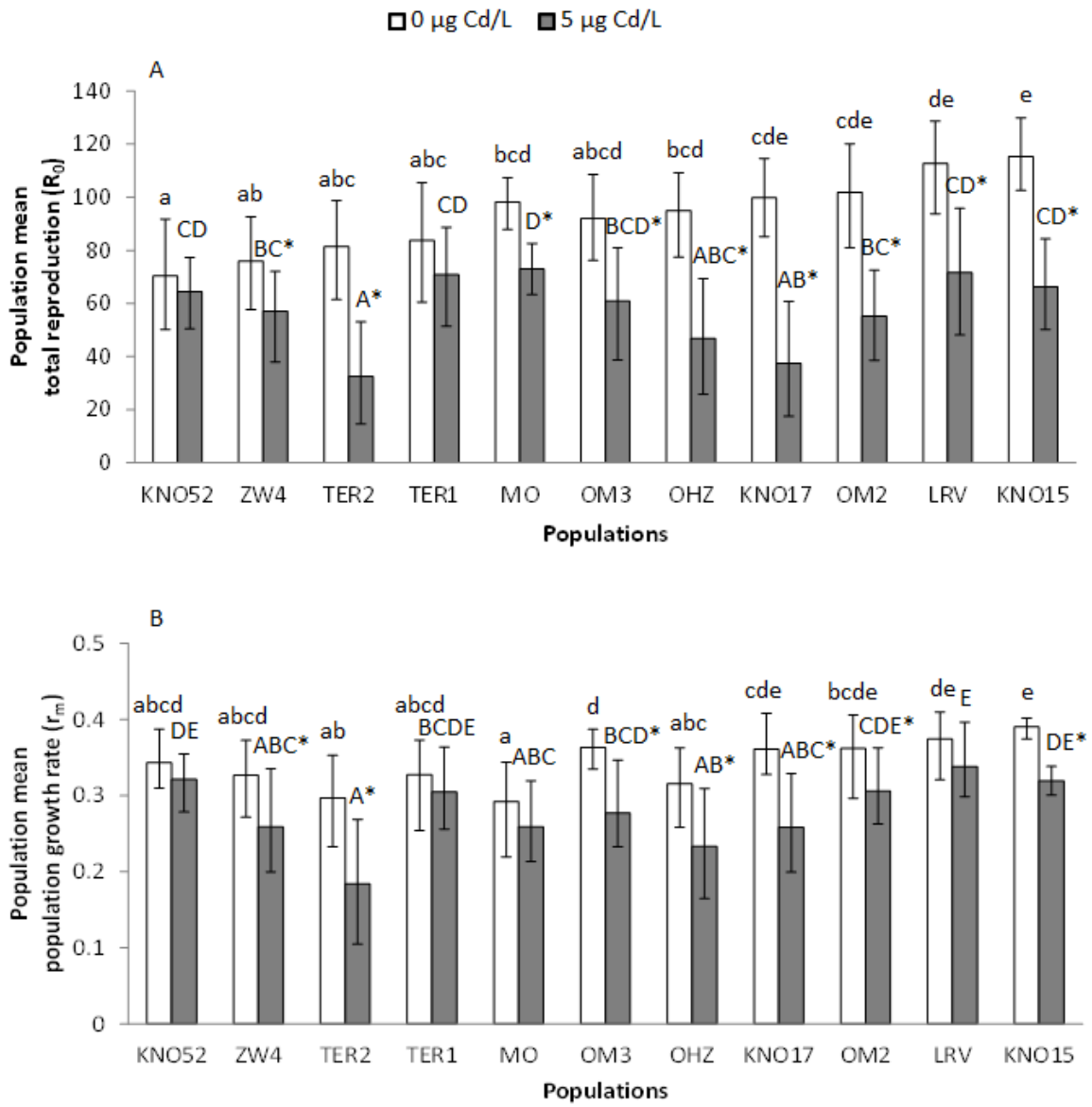

Figure 5.2: Median of population mean of total reproduction (R_0) (A) and population growth rate (r_m) (B) in the control (0 µg Cd/L) and Cd treatment (5 µg Cd/L). Error bars represent 95% confidence intervals. Populations that bear the same small letter are not significantly different from each other in the control treatment. Populations that bear the same capital letter are not significantly different from each other in the Cd treatment. An asterisk indicates a significant difference between control and Cd treatment per population.

Reproduction at first brood ranged between 7.0 and 16.5 juveniles per organism (Figure 5.3A). A population effect was found in control and Cd treatment (Table S5.110 -

Table S5.111). Non-parametric bootstrap resampling analysis (post-hoc analysis) indicated differences in population means between some populations as well in control as in Cd exposure. There was also a significant Cd effect for all populations (Figure 5.3A).

The population mean of maturation rate ranged between 0.10 (MO) and 0.13. There was a significant Cd effect for KNO52, OM2, OM3, ZW4 and LRV (Figure 5.3B). Despite a significant Cd effect on maturation rate for some populations, no significant population effects were found in control and Cd treatment (Table S5.112 and Table S5.113).

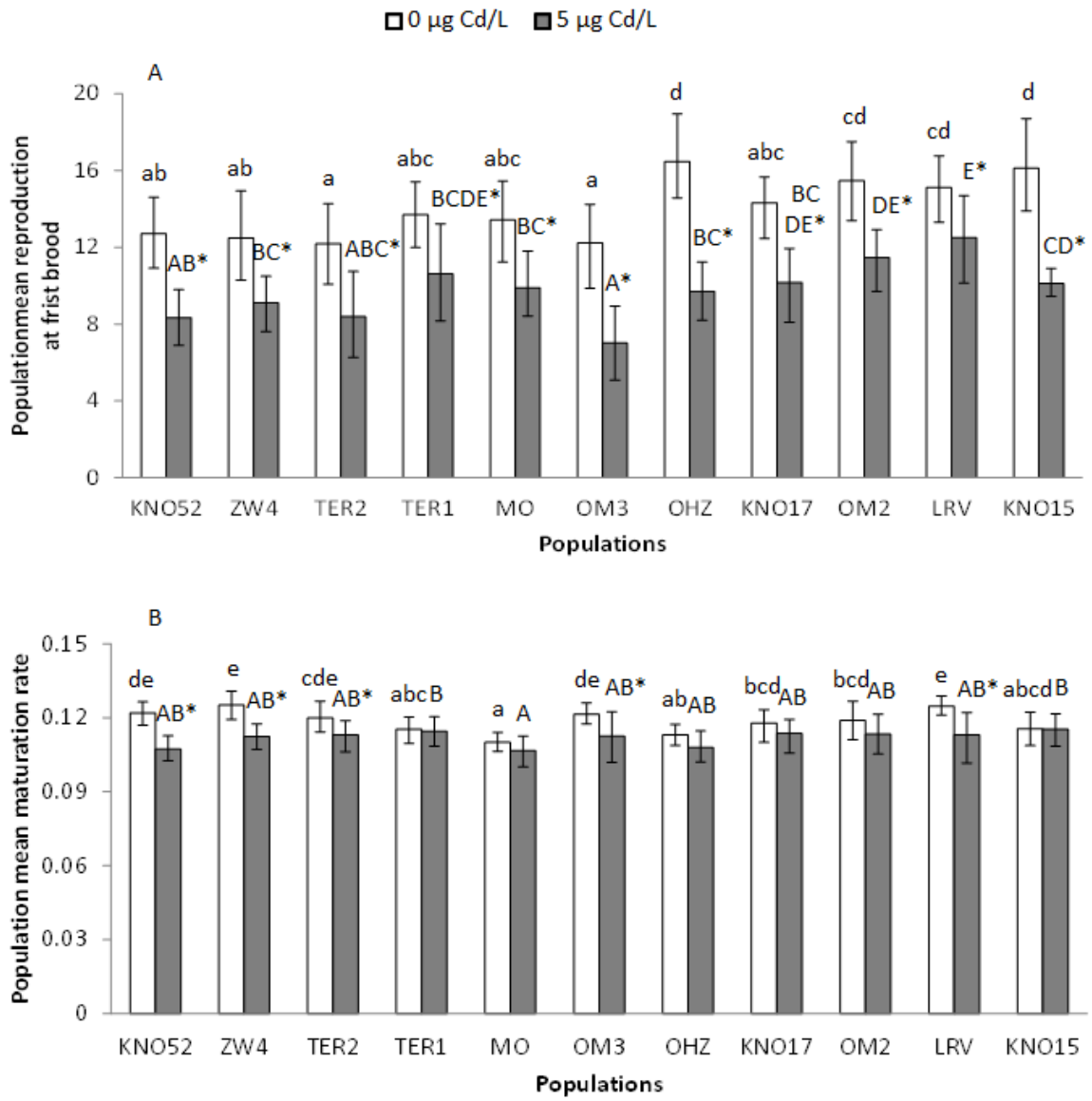

Figure 5.3: Median of population mean of reproduction at first brood (A) and maturation rate (B) in the control (0 µg Cd/L) and Cd treatment (5 µg Cd/L). Error bars represent 95% confidence intervals. Populations that bear the same small letter are not significantly different from each other in the control treatment. Populations that bear the same capital letter are not significantly different from each other in the Cd treatment. An asterisk indicates a significant difference between control and Cd treatment per population.

The population mean of Cd tolerance of total reproduction (R_0) was lowest in KNO17 (0.34), while in KNO52 the Cd tolerance of total reproduction (R_0) was highest (1.03) (Figure 5.4). The Cd tolerance of population growth rate ranged between 0.76 for KNO17 and 0.92 for MO (Figure 5.4). The population mean of Cd tolerance of reproduction at first brood ranged between 0.65 in OHZ and 1.10 in KNO17 (Figure 5.5). Maturation rate of Cd tolerance was highest in KNO15 (1.00) and lowest in KNO52 (0.90) (Figure 5.4). Generalized Linear

models indicated a significant population effect for total reproduction (R_0), population growth rate and maturation rate, but not for reproduction at first brood (Table S5.102 - Table S5.105). Non-parametric bootstrap-resampling indicated a between-population effect of Cd tolerance of the different fitness traits (Figure 5.4 - Figure 5.5).

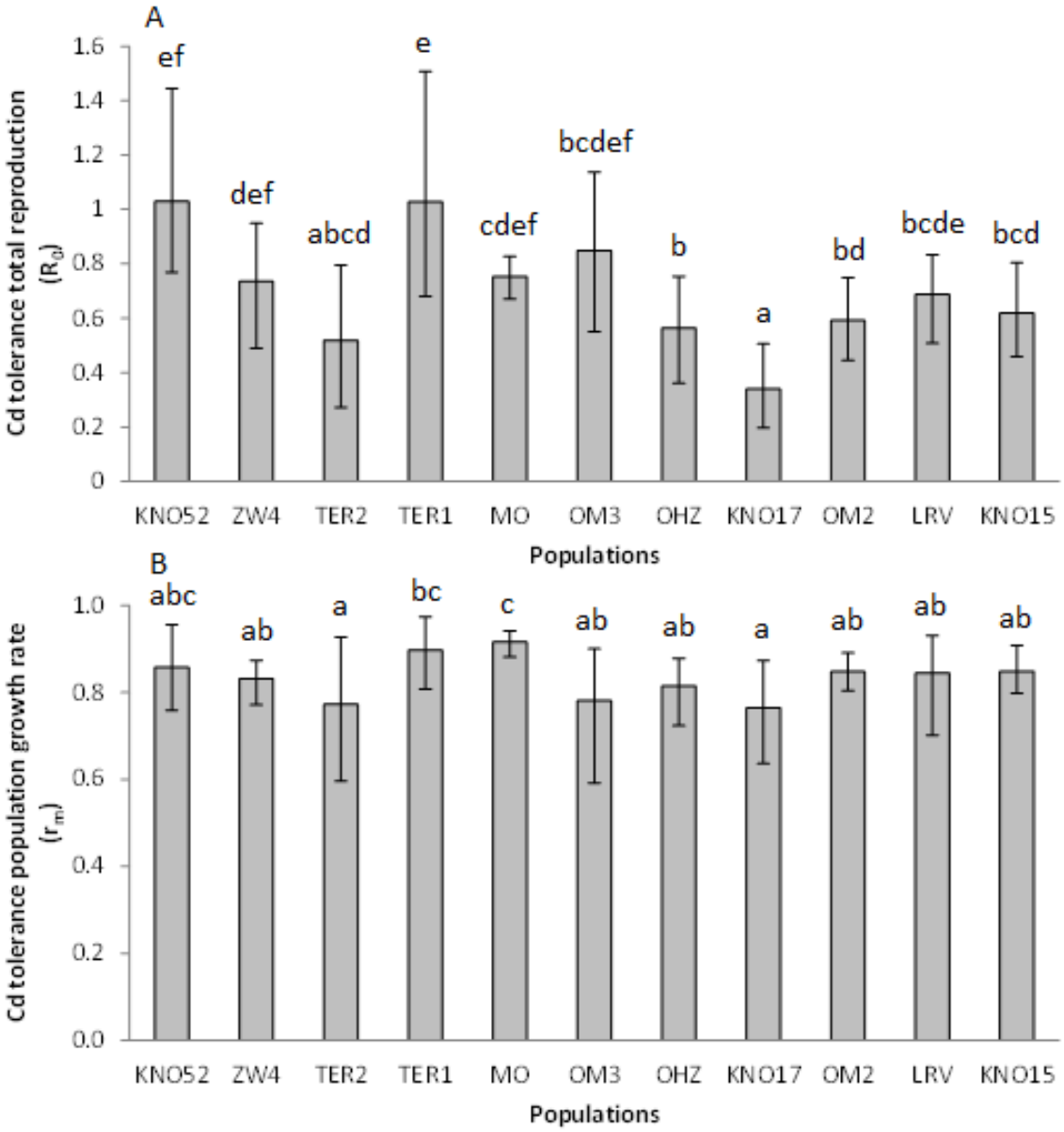

Figure 5.4: Median of population mean of Cd tolerance of total reproduction (R_0) (A) and population growth rate (r_m) (B). Error bars represent 95% confidence intervals. Populations that bear the same small letter are not significantly different from each other.

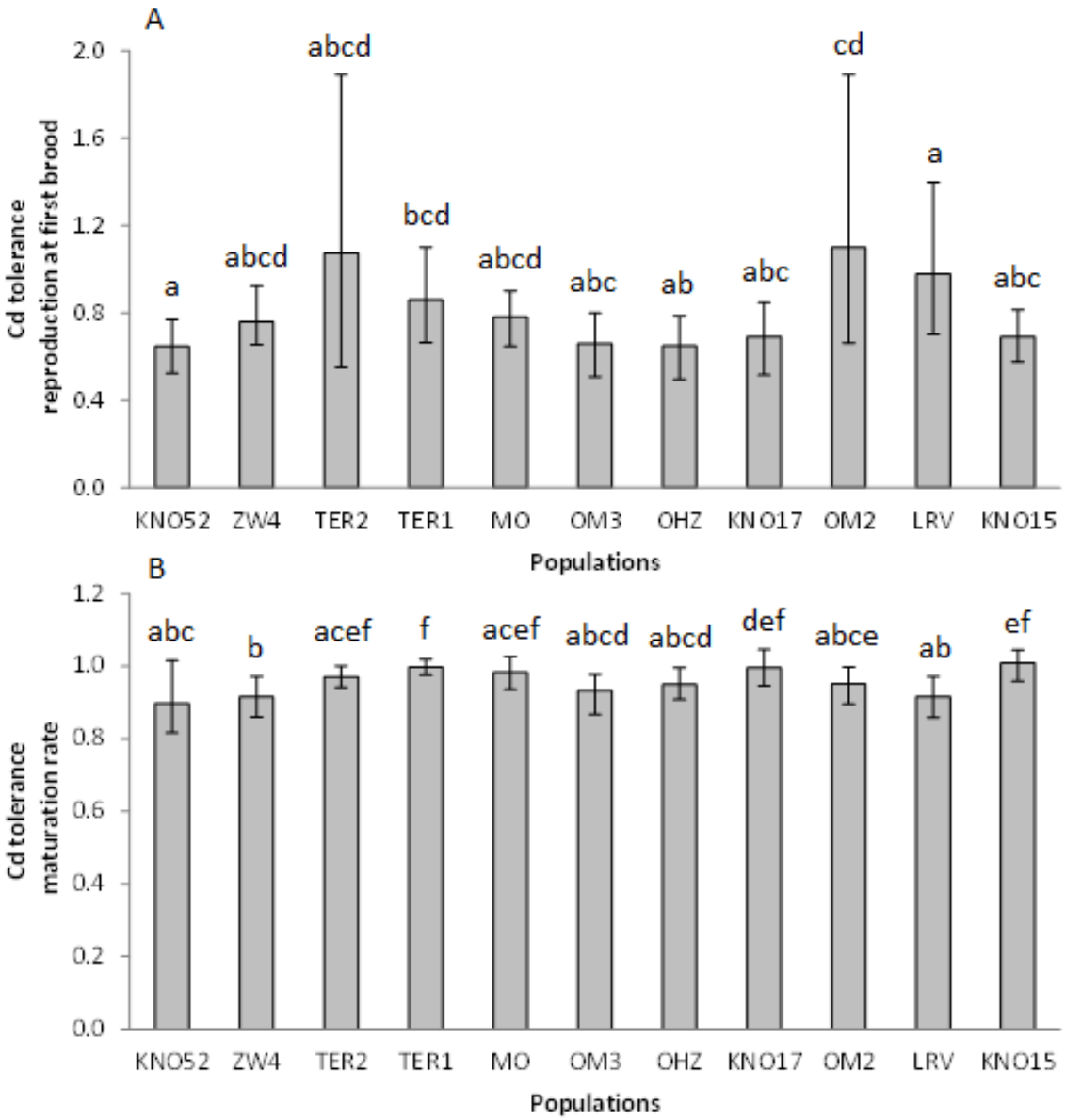

Figure 5.5: Median of population mean of Cd tolerance of reproduction at first brood (A) and maturation rate (B). Error bars represent 95% confidence intervals. Populations that bear the same small letter are not significantly different from each other.

3.3. Genetic coefficient of variation (CV_G) and broad sense heritability (H^2)

The genetic coefficient of variation of the different populations are summarized in Figure 5.6 - Figure 5.9. For total reproduction in the control treatment and Cd treatment (Figure 5.6), the genetic coefficient of variation (CV_G) and H^2 was not greater than 0 for 36% of populations in the control treatment and 72% in the Cd treatment. The CV_G ranged between 0 and 86.4%. The H^2 ranged between 0 and 0.79. There was a significant Cd effect

on H^2 in LRV, TER2, OHZ, OM3 and KNO17 (compared to the control). Post-hoc analysis by bootstrap resampling, indicated that not within all of the studied populations there was a substantial ($p<0.05$) genetic variability of total reproduction under Cd exposure (Figure 5.6 and Figure S5.2).

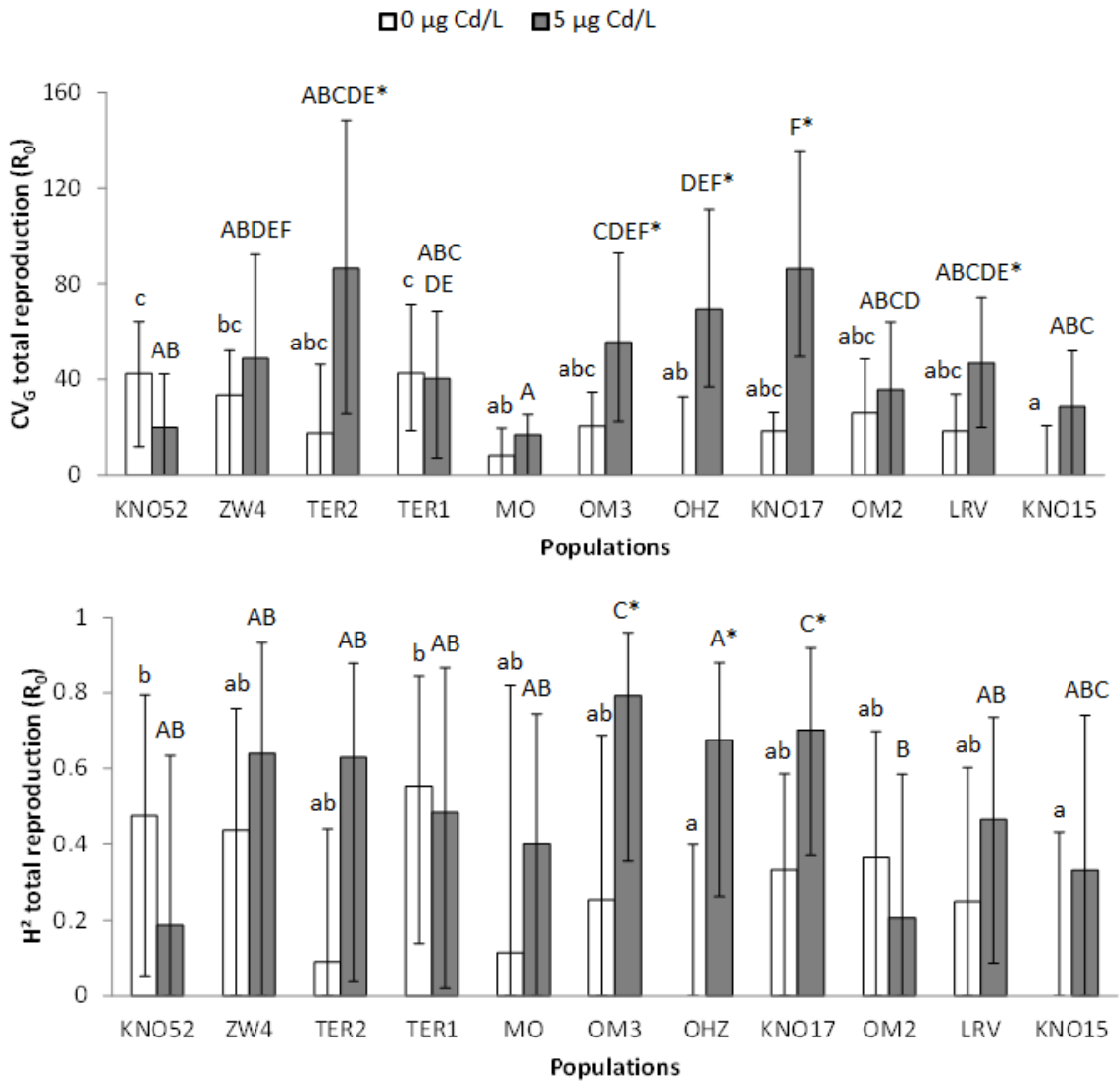

Figure 5.6: Median of genetic coefficient of variation (CV_G) and broad sense heritability (H^2) of total reproduction in the control (0 µg Cd/L) and Cd treatment (5 µg Cd/L). Error bars represent 95% confidence intervals. Populations that bear the same small letter are not significantly different from each other in the control treatment. Populations that bear the same capital letter are not significantly different from each other in the Cd treatment. An asterisk indicates a significant difference between control and Cd treatment per population.

The 5th percentile of CV_G and H^2 of r_m was not > 0 for 18% of the populations in the control treatment and for 55% in the Cd treatment. The median CV_G ranged between 0% and

69.01% (Figure 5.7). The medium H^2 ranged 0 and 0.75. There was a significant Cd effect observed, but not for all populations (Figure 5.7 and Figure S5.3).

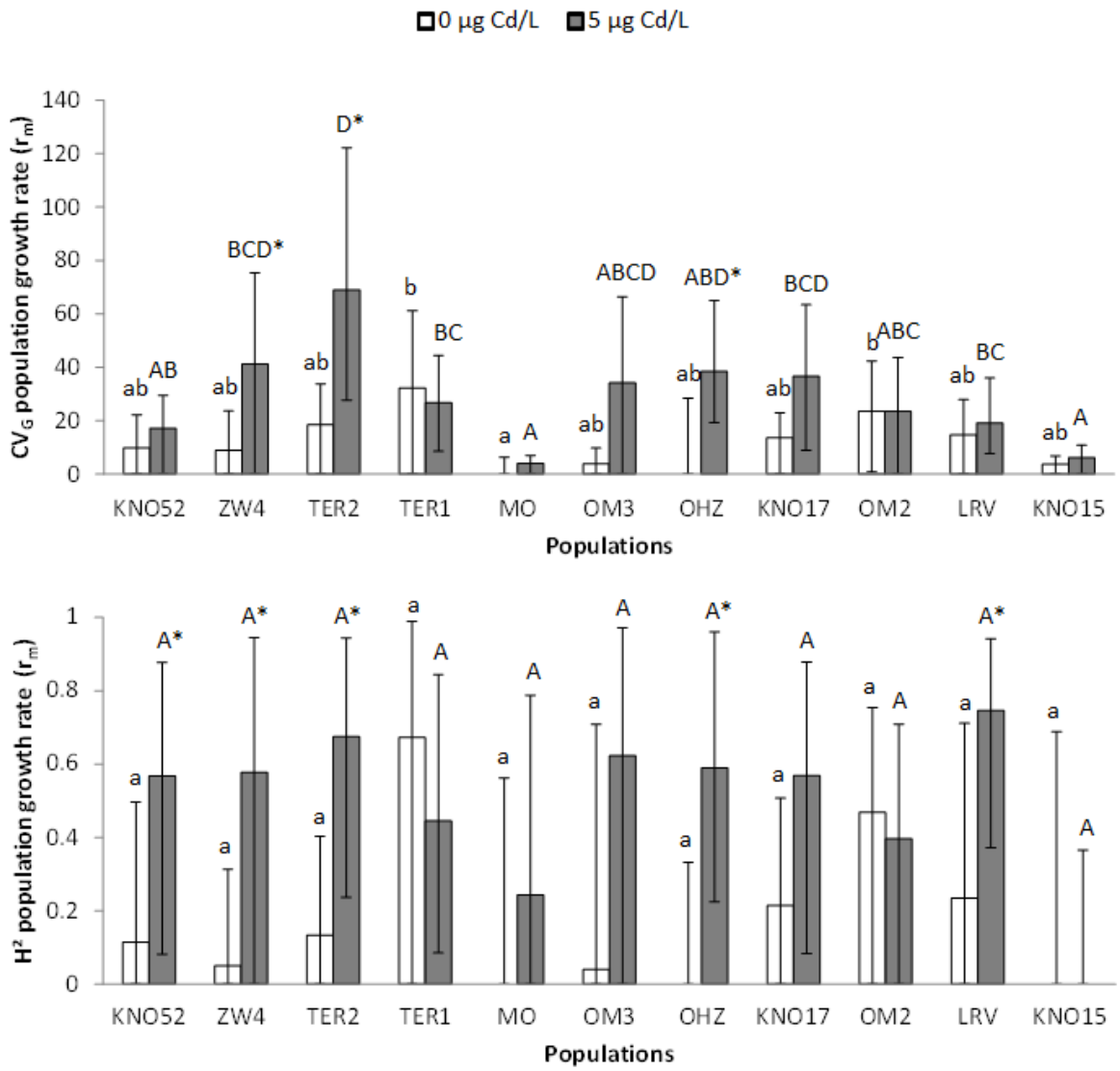

Figure 5.7: Median of genetic coefficient of variation (CV_G) and broad sense heritability (H^2) of population growth rate in the control (0 µg Cd/L) and Cd treatment (5 µg Cd/L). Error bars represent 95% confidence intervals. Populations that bear the same small letter are not significantly different from each other in the control treatment. Populations that bear the same capital letter are not significantly different from each other in the Cd treatment. An asterisk indicates a significant difference between control and Cd treatment per population.

The genetic coefficient of variation (CV_G) for reproduction at first brood ranged between 1.8% and 33.9% (Figure 5.8). There is only a significant difference between the two treatments for TER1. The median H^2 values for reproduction at first brood, ranged between 0.00 and 0.56 (Figure 5.8). For H^2, there was no Cd effect found. 18% of the populations in

the control treatment and 36% of the populations in the Cd treatment had a significant H^2 and CV_G.

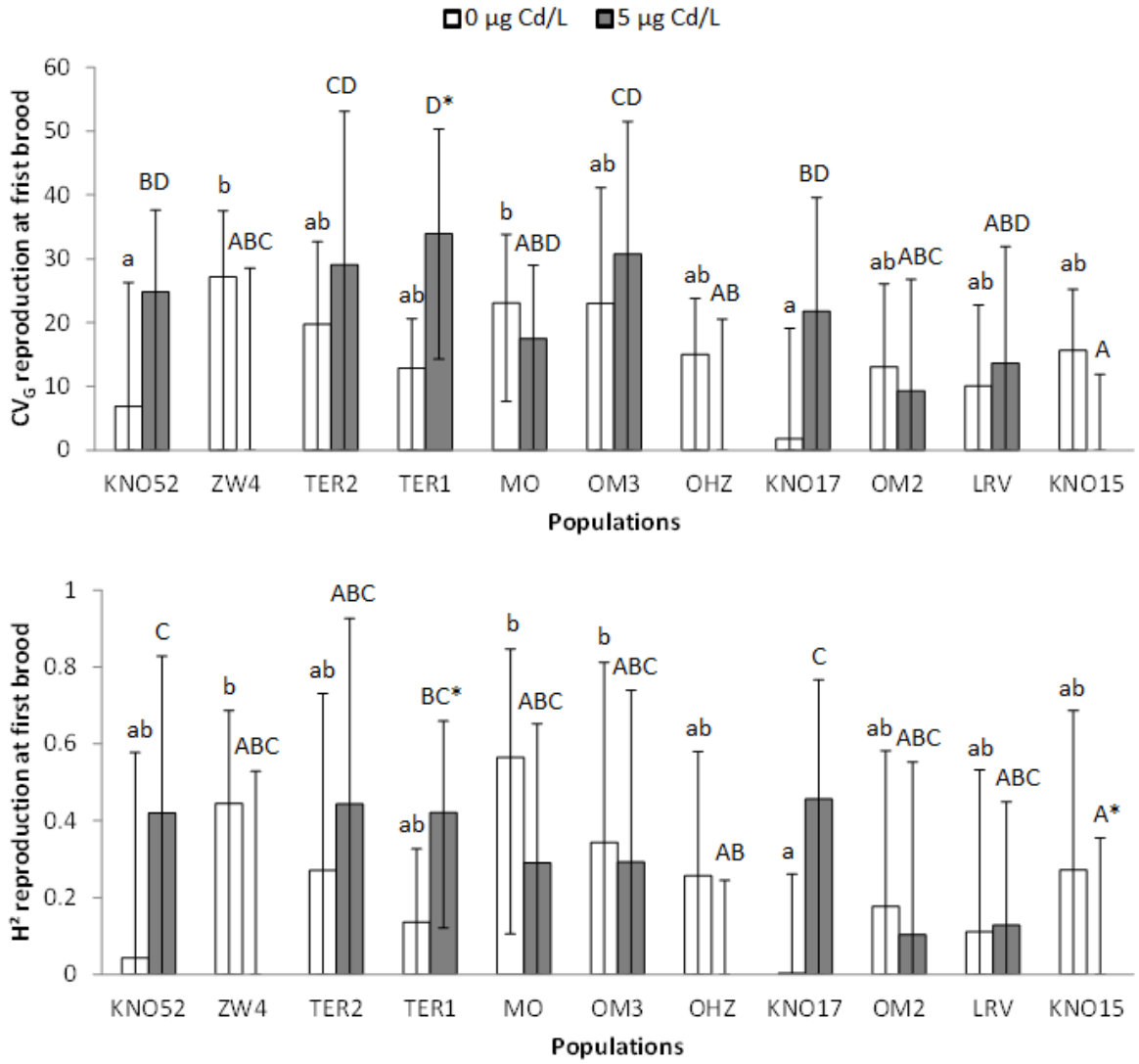

Figure 5.8: Median of genetic coefficient of variation (CV_G) and broad sense heritability (H^2) of reproduction at first brood in the control (0 µg Cd/L) and Cd treatment (5 µg Cd/L). Error bars represent 95% confidence intervals. Populations that bear the same small letter are not significantly different from each other in the control treatment. Populations that bear the same capital letter are not significantly different from each other in the Cd treatment. An asterisk indicates a significant difference between control and Cd treatment per population.

In terms of maturation rate, CV_G ranged between 0% and 11.99% (Figure 5.9). The 5th percentile was >0 for KNO52, OHZ, OM3, TER2. There was a significant difference between Cd and control treatment for LRV and OHZ. H^2 median values ranged between 0 and 0.66. Post-hoc analysis indicated no significant Cd effect for H^2. 63% of the populations in the Cd treatment had a significant CV_G and H^2.

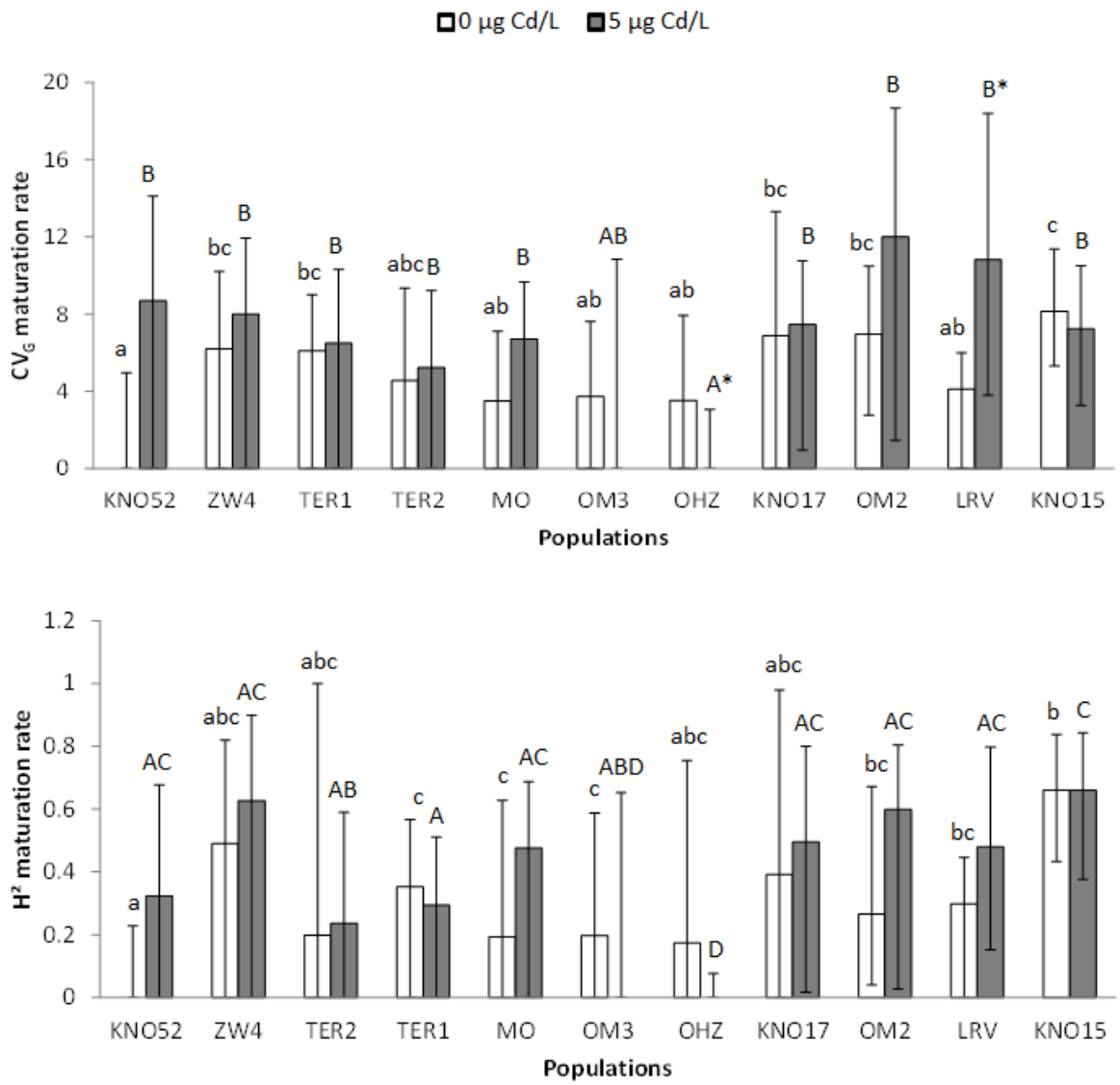

Figure 5.9: Median of genetic coefficient of variation (CV_G) and broad sense heritability (H^2) of maturation rate in the control (0 µg Cd/L) and Cd treatment (5 µg Cd/L). Error bars represent 95% confidence intervals. Populations that bear the same small letter are not significantly different from each other in the control treatment. Populations that bear the same capital letter are not significantly different from each other in the Cd treatment. An asterisk indicates a significant difference between control and Cd treatment per population.

3.4. Relationships between population parameters and habitat characteristics of the lakes.

Habitat characteristics are presented in Table S5.90-Table S5.93. The correlation coefficients are presented in Table S5.94 - Table S5.101. A summary can be found in Table 5.2. In general, several significant correlations were found between population means and metal concentrations in water and sediment.

Table 5.2: Summary of significant correlations between population means of traits and habitat-characteristics. + indicates a positive correlation was found. – indicates a negative correlation was found.

Trait	Correlation
R_0 (Cd treatment)	Ca_{water} (-)
r_m (Cd tolerance)	Pb_{water} (+) Ca_{water} (-)
Reproduction first brood (Cd tolerance)	$Pb_{sediment}$ (+) $Ni_{sediment}$ (+) Metal $ranking_{sediment}$ (+)
Reproduction first brood (Cd treatment)	$Cd_{sediment}$ (+)
Maturation rate (Control treatment)	Cd_{water} (+) *Vorticella* prevalence (-)
Maturation rate (Cd tolerance)	Parasite prevalence (+) Cd_{water} (+) Ni_{water} (+)

4. Discussion

Studies with various species suggest that environmental stress may increase evolutionary rates by increasing the level of genetic variability in life-history traits (Hoffmann and Parsons, 1991; Barata et al., 2002 a,b). A higher CV_G and/or H^2 thus suggests a higher micro-evolutionary potential. However, our results show that not all traits and not all populations exhibited a significant CV_G and/or H^2 under Cd stress (5 µg Cd/L) which indicates that there is no significant evolutionary potential for populations for some of the fitness traits under Cd stress. For total reproduction (R_0), 72% of the populations had a significant CV_G and H^2 (Figure 5.6). In our first hypothesis, we stated that Cd exposed populations would exhibit more genetic variation for life-history traits than the control population (expressed as H^2 and/or CV_G). For total reproduction (R_0), a significant effect was found between control and Cd for 45% and 27% of the populations (see Figure 5.6), for CV_G and H^2 respectively. For population growth rate, a significant effect was found for 27% and 45% of the populations

for CV_G and H^2, respectively. The studied Blankaart population of the chapter 4 showed no significant effect of exposure to 4.3 µg Cd/L (actual Cd concentration) on the CV_G for any of the traits (at 20°C).

For total reproduction (R_0), the largest Cd effect on population mean was found for the populations TER2 and KNO17 (-60% and -62% effect) (Figure 5.2). This was accompanied with a larger increase (factor 3 to 4) in CV_G and H^2 under Cd stress compared to the control treatment (Figure 5.6). The smallest effect was found in the KNO52 population (-8%), accompanied with a CV_G and H^2 which was not different in the Cd and control treatment. Similar results were found for population growth rate (r_m) (Figure 5.7). Thus, the hypothesis proposed by Hoffmann and Parsons (1991), i.e. that increased stress (in this case Cd stress) is expected to result in increased genetic variation, is actually supported by the combined results of the present, the previous chapters (chapter 2 and 3) and the Barata et al. (2000b) study (see chapter 2 for in-depth discussion on the latter study). Nonparametric bootstrap analysis indicated that there are between-population differences of the population means of Cd tolerance, and of several fitness traits in the control and the Cd treatment (Figure 5.2 - Figure 5.5). Hence, our results indicate that there are differences between population responses to Cd stress (hypothesis 2). According to Hoffmann and Parsons (1991), the tolerance of populations to toxic stress is inversely related to selective pressures experienced in their local habitats leading to local adaptation. On the other hand, genetic variability in tolerance within populations is positively related with the level of stress. As the populations in this study originated from pristine habitats that are not impacted by Cd, the populations would be expected to show similar levels of tolerance to Cd. Yet, this was not observed (Figure 5.4 - 5.5). This is in contrast with Barata et al. (2002a), who did not find a

significant difference in EC_{10} of individual fitness ($\lambda=e^{(clutch\ size/\ time\ to\ first\ brood)}$) of Cd between three *D. magna* populations originating from pristine environments. Our results show that there is, although not for all populations, significantly higher (within population) genetic variability under Cd stress (compared to the control). There are differences between populations in terms of CV_G and H^2 (hypothesis 3). This is in contrast with Agra et al. (2010), who observed similar levels of heritability (H^2) levels in tolerance to Cu and Zn in a reference and impacted population, however only two populations were studied (in contrast with the 11 populations studied in this chapter).

As the populations in this study were all tested simultaneously under the same conditions, differences in bio-availability, food regimes, etc. during tests cannot explain the observed tolerance differences between the populations. Differences in genetic composition and the evolutionary history of these 11 populations (prior to their collection in the field) may, however, also have contributed to the sensitivity differences. In this context (Table S5.13 - Table S5.14 and Table S5.90 – Table S5.93), some positive and negative correlations of Cd tolerance with habitat characterstics were observed, although these correlations were rather weak. The tolerance of reproduction at first brood was positively correlated with the metal content ranking of the sediments. So it seems that the more tolerant populations for Cd were found in the lakes with the highest metal concentrations in the sediment although no severe metal contamination in any of the lakes was noted. Coors et al. (2009) found a correlation between land-use and carbaryl tolerance. The observed positive correlation between EC_{50} values for carbaryl and land use intensitiy suggested a local adaptation for tolerance to such pesticides. A correlation between land-use and potassium dichromate tolerance was not found, as this toxicant is not related to crop cultivation (Coors et al.,

2009). This finding is in agreement with our observations (no consistent correlation with land-use either). Also Lopes et al. (2005) found similar resuls. The results indicated that individuals from impacted populations presented a higher tolerance (expressed as cumulative mortality) to very toxic and moderately toxic water (water originating from AMD impacted habitats) compared to reference populations. As indicated before, these studies compared reference populations and impacted populations. In contrast, our study focused only on populations originating from non Cd contaminated environments, although these populations were impacted with other stressors (land-use, fish abundance, parasite-presence).

In conventional risk assessment, monoclonal laboratory populations of *Daphnia magna* are commonly used to determine potential risks of toxicants. For Cd tolerance of total reproduction at 5 µg Cd/L a factor 3 difference between most sensitive and tolerant population was found. Based on clonal reproduction, Cd tolerance of total reproduction ranged between 0 and 2.15. Barata et al. (2000a) studied feeding responses of laboratory *D. magna* clones (to Cu, Cd and fluoranthene) and reported differences in EC_{10} and EC_{50} of about 4-fold difference. Also for other species, e.g. *Potamopurgus antipodarum,* a 3-fold difference between most sensitive and tolerant clone for acute Cd toxicity (LC_{50}) was found (Jensen and Forbes, 2001). In the EU, when chronic NOECs for a chemical are available for three trophic levels (typically an alga, a fish and *Daphnia* sp.) the lowest NOEC (most sensitive species) is conventionally divided by an assessment factor of 10 to obtain the PNEC or EQS. These concentrations are considered to have 'no effects' on freshwater populations, communities and ecosystems. When a large number of tests are available, distribution based extrapolation models are used to estimate environmental risks. For many chemicals, *D.*

magna is the most sensitive species tested (Wogram and Lies 2001; Von der Ohe and Lies, 2004) and for many chemicals a NOEC will only be available for a single *D. magna* clone. In the present study, we showed genetic variability among and within *Daphnia magna* populations. According to Forbes (1998) the range and distributions of tolerance in field populations should be used to quantify the extent to which genetic variability should be incorporated in risk assessment.

5. Conclusion

We have examined the variability between and within population responses of 11 *Daphnia magna* populations exposed to Cd stress originating from pristine habitats (in terms of Cd contamination). We observed significant differences in Cd tolerance of different fitness traits between populations. Although not all populations exhibited a significant CV_G and or H^2 (64% in control treatment and 28% in Cd treatment), for some populations there were differences in micro-evolutionary potential under Cd stress (compared to the control). For total reproduction (R_0), 45% and 27% of the populations had a significantly higher CV_G or H^2 respectively compared to the control. These observed differences in micro-evolutionary potential under Cd stress (increased CV_G and/or H^2 compared to the control) may eventually lead to a different reduction of clonal diversity (compared to a control) in a natural setting. Our results suggest that genetic differences within and between populations in tolerance to toxicant exposure should be considered in the ecological risk assessment process of chemicals.

Chapter 6: Micro-evolutionary response in a *Daphnia magna* population exposed to Cd stress under semi-field conditions

Abstract- A 203 days during micro-evolutionary experiment was conducted to test the micro-evolutionary response in a *Daphnia magna* population exposed to a control and Cd range between 2.11 and 20.77 µg Cd/L under semi-field conditions. In a following life-table experiment, clones (or isolates) from the original population (= start population), the long-term Cd exposed population and the long-term control exposed population were tested under a control and Cd concentration between 2.02 µg Cd/L and 17.83 µg Cd/L. Total reproduction (R_0) and population growth rate (r_m) were monitored during 21 days. Our results indicate that on population level, there was a higher fitness observed at 17.83 µg Cd/L in the long-term 20.77 µg Cd/L exposed population compared to the long-term control exposed population and the start population. However, general linear model analysis indicated a significant aquaria effect, so genetic drift could not be excluded in the analysis of our results.

1. Introduction

Questioning whether populations adapt to contamination is critical for environmental risk assessment (Chaumot et al., 2009). If the potential for adaptation is not considered, then the long-term ecological risks may be overestimated (Millward and Klerks, 2002) although a negative consequence of adaptation can be accompanied by fitness costs or decreased genetic variations (Ward and Robinson, 2005). Chemical contamination can alter genetic diversity through genetic bottleneck effects and/or contaminant-induced selection (Van Straalen and Timmermans, 2002). The ability of populations to survive in metal contaminated habitats has been widely reported, as shown in studies of Lopes et al. (2004, 2005); Agra et al. (2010); Klerks (2002). Those studies indicate that organisms that originated from a contaminated habitat had higher tolerance than those from uncontaminated site populations. The changes in gene frequencies in the populations can involve (1) the elimination of sensitive individuals, which would lead the population to genetic erosion, (2) the appearance of a new gene through mutations or (3) a new combination of genes (sexual reproduction) underlying a new or more efficient tolerance mechanism followed by their increased frequency by natural selection (Lopes et al., 2006). By comparing populations from polluted sites and reference sites, it is not possible to fully exclude that such sites differ in characteristics other than the pollutant of interest and hence casually relate the increased tolerance to the historic exposure to pollution (Xie and Klerks, 2003). Experimental evolution is a powerful tool for evolutionary ecologists to study the genetic response of organisms to selection pressures and thus to investigate whether organisms are able to adapt to environmental change (Conner, 2003). In the present study we used this approach to investigate the presence of micro-evolutionary responses in a

Daphnia magna population exposed to a Cd concentration range between 0 and 22 µg Cd/L under semi-field conditions. The populations exposed to Cd were allowed to evolve 'naturally' for 203 days, i.e. to undergo natural selection. Indeed those clones having the highest fitness under Cd stress would be expected to increase their frequency under such long-term Cd exposure, thus resulting in an increase of the mean fitness of the population in that aquarium with time (hypothesis 1). Thus we investigated if the long-term exposures to Cd (2.2-22 µgCd/L) had a higher fitness in the Cd treatment than in the long-term control exposures and start population. This type of experimental evolution studies is a commonly used tool in evolutionary ecology (Van Doorslaer et al., 2007; Van Doorslaer et al., 2010; Cousyn et al., 2001) but heavily underutilized in ecotoxicology, with few exceptions (e.g. Ward and Robinson, 2005; Jansen et al., 2010; Brausch and Smith, 2009; Xie and Klerks, 2003; Lopes et al., 2009).

Another factor influencing long-term consequences of evolution of increased fitness under Cd stress is the presence of a cost of tolerance. The evolution of increased tolerance to pollution may be important for ecological risk assessment because (1) it may allow the persistence of populations in contaminated habitats and (2) it may lead to a reduction in genetic diversity (Lynch and Walsh, 1998). This in turn may lead to a decreased tolerance to other stressors (Ward and Robinson, 2005), a reduced adaptive potential towards future challenges imposed by novel stressors (Van Straalen and Timmermans, 2002) or a reduced fitness when the selective pressure is removed (e.g. after remediation of a polluted site), an observation which is commonly referred to as "cost of tolerance" (Medina et al., 2007). This phenomenon is caused by genetic between-environment correlations or between-environment trade-offs (Medina et al., 2007). For example, Shirley and Sibly (1999) observed

that a *Drosophila* population cultured under high Cd stress during several generations exhibited lower reproduction when reared in clean media afterwards. Similarly Postma et al. (1995a) showed that Cd tolerant *Chironomus riparius* populations had lower fitness when reared in a clean environment. Levinton et al. (2003) indicated that after clean-up of a Cd polluted site, the loss of tolerance in *L. Hoffmeisteri* had a genetic basis. Although knowledge of between-environment trade-offs is considered a key element for incorporating micro-evolution in the environmental risk assessment paradigm (Medina et al., 2007), this type of limited information is only available for highly contaminated environments (see reviews in Medina et al., 2007 and Morgan et al., 2007). Knowledge of such trade-offs in contaminated systems with a range of Cd concentrations is completely lacking. Irrespective of whether micro-evolutionary responses under chemical stress are considered "positive" or "negative", it is of interest to know how the micro-evolutionary potential is affected as a function of chemical concentration (sublethal concentrations versus high concentrations).

2. Material and Method

2.1. Micro-evolutionary experiment

In December 2009, a micro-evolution experiment was initiated involving a *Daphnia magna* population consisting out of 123 clones exposed to a Cd concentration range of 0-22 µg Cd/L. Each Cd treatment was replicated three times, resulting in a total of 15 aquaria. The 123 *Daphnia magna* clones originated from ephippia collected in November 2009 from the Kasteelvijver pond in the nature reserve Blankaart (Diksmuide, Belgium) using a Van Veen grab and a sediment corer. The ephippia were hatched as described in Chapter 2 (§2.1.) and a single hatchling was selected to establish a clonal lineage. The juvenile hatchlings were transferred to 50 mL polyethylene cups. The maintenance of this first generation (=P-

generation Figure 6.1) was the same as described in Chapter 2. Four juveniles from the third brood of each clone of this P-generation were selected and put individually in 50 mL ethylene cups with modified M4 medium (= F1-generation, Figure 6.1). Fifteen juveniles (=F2-generation) from the third brood of one of the four Daphnids of the F1-generation were selected and put individually in 5L aquaria filled with 4L modified M4-medium and a Cd concentration (between 0 and 22 µg Cd/L). So each clone was present in each aquarium. In total 123 clones were put in each aquarium. The experiment lasted for 203 days. The aquaria were renewed every week, and with every renewal, a culling regime of 20% was conducted. This culling regime is based on experiments of Van Doorslaer et al. (2009). Organisms collected through this culling regime were counted, which gives an indication of population density of daphnids in the aquaria. Also, with every medium renewal, ephippia were counted and collected. Daphnids were fed daily with *Pseudokirchneriella subcapitata* and food density in the aquaria was daily adjusted according to a seasonal pattern. This seasonal pattern was based on the study of Muylaert et al. (2003), i.e. the average of the phytoplankton concentration in Lake Blankaart in 1998 and 1999. With every medium renewal, ephippia were collected and stored in dark at 4°C. The water temperature over the days of the experiment followed a sinusoidal pattern starting at 10°C, which is the water temperature in spring (Arbaciauskus and Lampert, 2003). The water temperature was regulated with a water cooler (TECO, Ravanna, Italy). During the micro-evolutionary experiment, DOC and Cd samples were taken on a weekly basis.

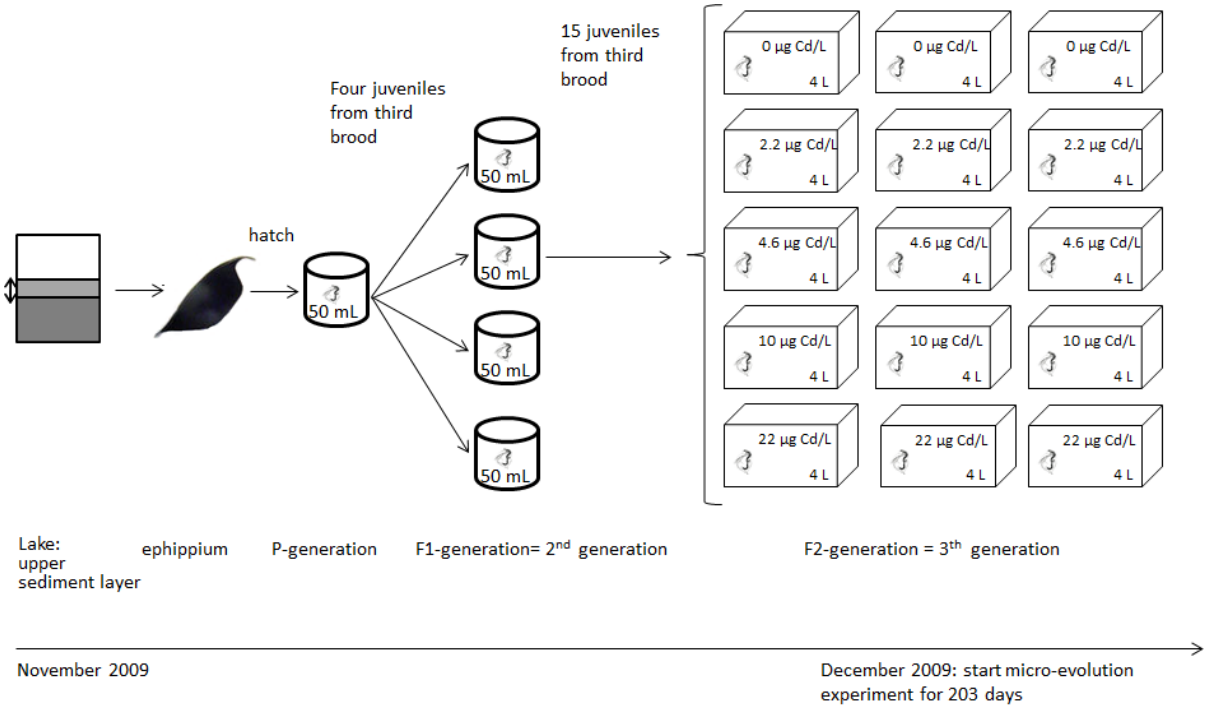

Figure 6.1: Schematic overview of experimental design for one clone originating from field population. The same design was followed for all 123 clones.

2.2. Life-table experiment

The test design is scheduled in Figure 6.2A-B-C. In a first step, 5 random picked juveniles from each aquaria (i.e. 15 in total for each Cd concentration between 0 and 22 µg Cd/L) and one juvenile randomly chosen from 12 clones kept under laboratory population (= start population) were transferred to separate 50 mL polyethylene beakers (= P-generation in Figure 6.2.A-B-C) in modified M4 medium (see chapter 2,§2.1). For each isolate, one juvenile (<24h) from the third brood was randomly picked out, to start the second generation (2nd generation, i.e. F1, in Figure 6.2.A-B-C). Each juvenile was transferred individually to a separate 50 mL polyethylene beaker. The individuals in this second generation (F1) then served as the mothers for producing the following generation. For the start and long-term control exposed population, six juveniles (<24h) (F2) from each of the three mother organisms (F1) were selected and were placed individually in 50 mL polyethylene vessels with modified M4 medium and with a Cd range between 2.2 and 22 µg

Cd/L (added as $CdCl_2 \cdot H_2O$) including a control (no added Cd). For the long-term Cd exposed population, two juveniles (<24h) from each of the three mother organisms were selected and were placed individually in 50 mL polyethylene vessels with modified M4 medium and the Cd concentration exposed during the micro-evolution experiment and the control treatment. For example, from one mother organism, one juvenile from the long-term 2.2 Cd/L exposed population was put in a control treatment and one juvenile was put in a 2.2 µg Cd/L. As such, maternal effects can be ruled out, as for each clone in each Cd concentration, each of the replicate individuals (juveniles) being exposed originated from a different mother organism. All Cd exposures with all clones were simultaneously initiated, allowing a comparison that is not biased by temporal variability of the cultures. Medium renewal was three times a week and organisms were fed daily with 250 µg dry wt/individual, 500 µg dry wt/individual and 750 µg dry wt/individual in the first, second and third week of their life, respectively. Based on daily observations the following traits were determined: population growth rate (r_m), survival and total reproduction at day 21 (R_0). pH was measured at every renewal of the old medium per Cd concentration and in the beginning of the test of the new medium.

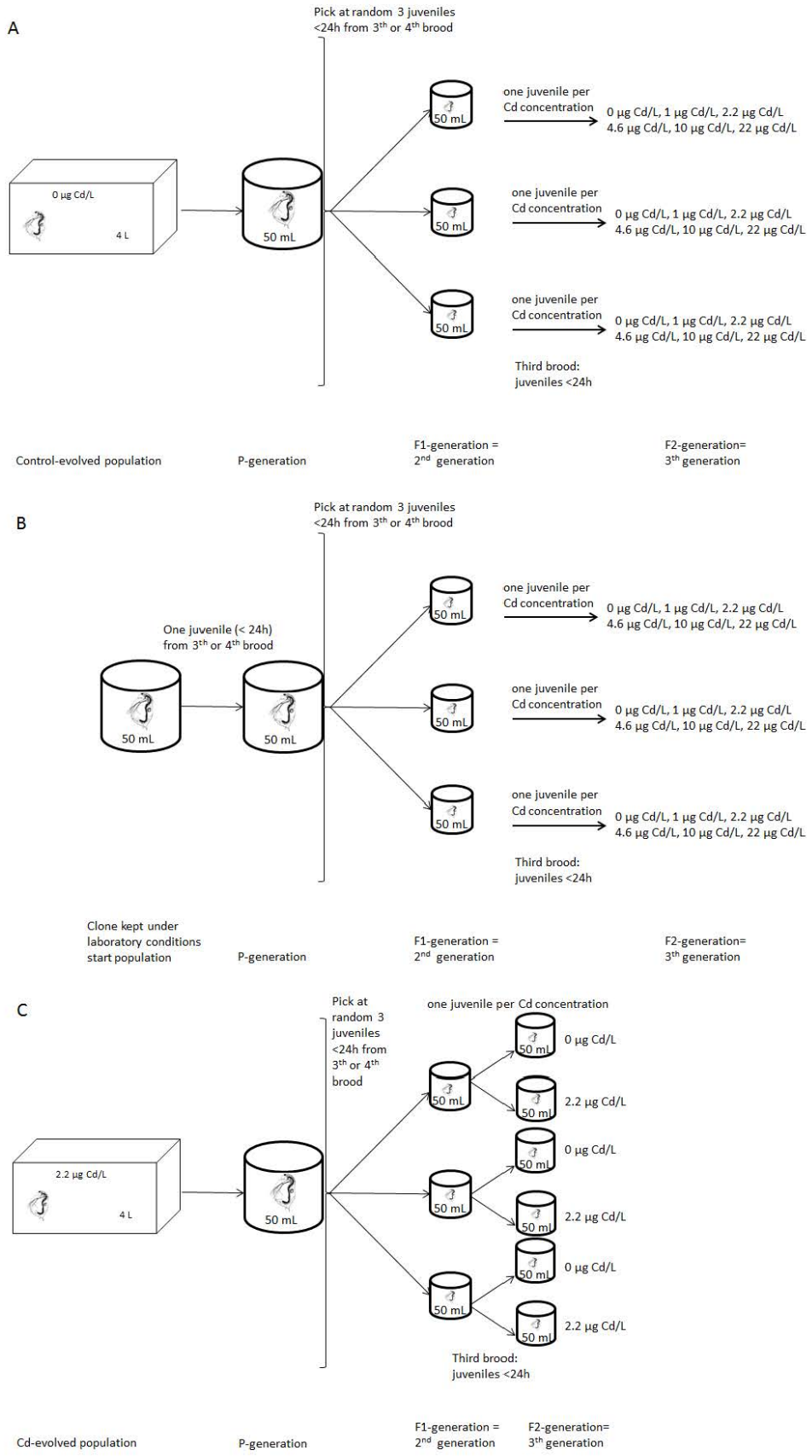

Figure 6.2: Test design for long-term control exposed population (A), start population (B) and long-term Cd exposed populations (C).

2.3. Chemical analyses

Chemical analyses were performed as described in chapter 2 (§2.4).

2.4. Statistical analyses

Calculation of population means is the same as described in chapter 2 (§2.5). The median values (50th percentile) and the 2.5th and 97.5th percentile of population means (??? are reported. The population mean in a treatment of the long-term Cd exposed population was considered significantly higher than the population mean in the same treatment of the long-term control exposed population and start population if more than 95% of the calculations yielded $\overline{X}$ (long-term Cd exposed) > $\overline{X}$ (long-term control exposed) and $\overline{X}$ (start population) (i.e. equivalent to a one-sided test at the 0.05 significance level). All calculations were performed in MATLAB 7.5.0.342 (Mathworks Inc) software.

To rule out an aquaria-effect (i.e. three replicates per long-term Cd exposed population) within the long-term exposed populations, among-aquaria variability in sensitivity to Cd stress was assessed from through a General Linear Model (see detailed information on GLM in Supplementary Material S6.1.) on the different fitness traits considering aquaria as fixed factor and clone nested in aquaria as random. To determine the long-term exposure effect on the long-term control and Cd exposed populations under tested Cd concentrations, General Linear Model analysis was performed on total reproduction and population growth rate with long-term exposure as fixed factor, aquaria (nested in long-term exposure) and clone (nested in aquaria) as random factors. Analyses were performed using Statistica 7.0 (Statsoft, Tulsa, OK).

Differences in ephippia production between the long-term Cd exposed populations (0 - 22 µg Cd/L) were detected using one-way ANOVA and post-hoc Duncan test. Analyses were performed using Statistica 7.0 (Statsoft, Tulsa, OK). At every medium renewal (i.e. time-point in the micro-evolutionary experiment), the density of the several *Daphnia magna* populations in the different aquaria was also determined. At each time point, a Kruskall-wallis test was performed to determine significant differences between the populations ($p<0.05$). These analyses were performed using Statistica 7.0 (Statsoft, Tulsa, OK).

3. Results

3.1. Physico-chemical measurements

The physico-chemistry of the test media and in the aquaria is presented as supportive information (Table S6.1 - Table S6.2). DOC ranged between 4.1 and 5.9 mg/L in the Cd experiment and between 4.6 and 6.0 mg/L in the micro-evolution experiment. pH ranged between 7.6 and 7.9. The mean dissolved Cd concentrations (mean of old and new medium) differed at most 23% and 9% from the nominal Cd concentration in the Cd experiment and in the micro-evolutionary experiment respectively.

3.2. Micro-evolutionary responses

Values of individuals are presented in Supplementary Material (Table S6.3 –Table S6.39). Results of population means are presented in Figure 6.3 and Figure 6.4. The long-term 20.77 µg Cd/L exposed population had a significant larger population growth rate (r_m) and significant larger total reproduction (R_0) compared to the start and the long-term control exposed population tested in 17.83 µg Cd/L. In the control treatment, the long-term 2.11 µg Cd/L exposed population had significantly higher reproduction (R_0) in control treatment than

the start population and the long-term control exposed population. No costs for adaptation was found, as no significant lower fitness of the long-term Cd exposed populations in the control treatment was found compared to the long-term control exposed population and start population (Figure 6.3 and Figure 6.4).

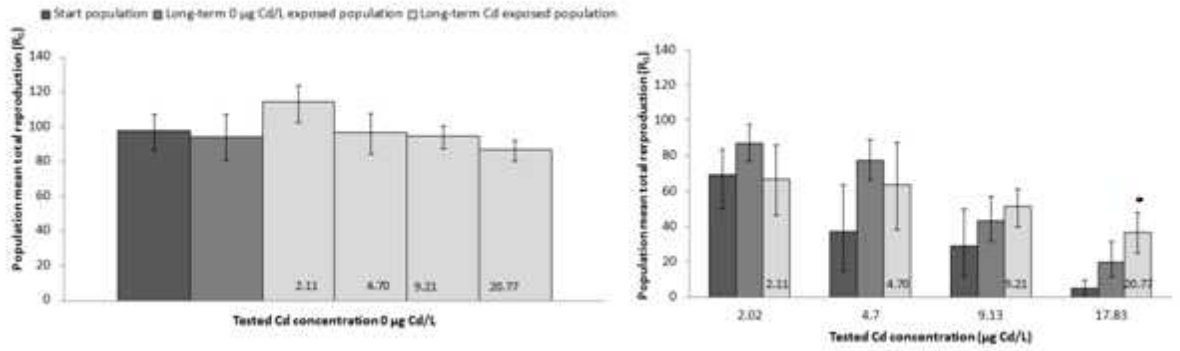

Figure 6.3: Results of the 203-day experimental evolution experiment, total reproduction (R_0) in control treatment (A) and Cd treatments (B) are reported. Values presented in bars of long-term Cd exposed population represent measured Cd concentrations to which the long-term Cd exposed populations have been exposed during the entire experimental evolution study. An asterisk indicates a significant difference between the long-term Cd exposed population and both the long-term control exposed and start population. Error bars represent 95% confidence intervals. The measured Cd concentrations during the evolution experiment in the long-term control exposed population was <0.1 µg Cd/L. The measured Cd concentration of the start population during maintenance of the start population under laboratory conditions was <0.1 µg Cd/L.

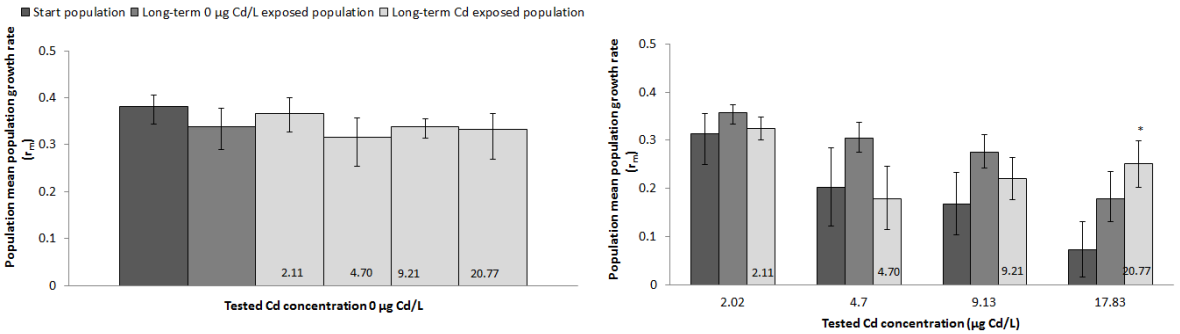

Figure 6.4: Results of the 203-day experimental evolution experiment, population growth rate (r_m) in control treatment (A) and Cd treatments (B) are reported. Values presented in bars of long-term Cd exposed population represent measured Cd concentrations to which the long-term Cd exposed population have been exposed during the entire experimental evolution study. An asterisk indicates a significant difference between the long-term Cd exposed population and both the long-term control exposed and start population. Error bars represent 95% confidence intervals. The measured Cd concentrations during the evolution experiment in the long-term control exposed population was <0.1 µg Cd/L. The measured Cd concentration of the start population during maintenance of the start population under laboratory conditions was <0.1 µg Cd/L.

General Linear Model analysis indicated an aquaria effect in the long-term control exposed population exposed to 9.19 µg Cd/L for total reproduction (R_0) (Table 6.1) and in the

long-term control exposed to 17.83 µg Cd/L for population growth rate (r_m) (Table 6.2). In the long-term Cd exposed populations no aquaria effect was found. Clone effects were found in the long-term 20.77 µg Cd/L and 4.7 µg Cd/L exposed population for total reproduction (R_0) and population growth rate (r_m) (Table 6.1 and Table 6.2). Individual values were log(x+1) transformed.

Table 6.1: General Linear Model for effects in the long-term Cd exposed populations with aquaria (fixed factor) and clone effect nested in aquaria (random factor) on total reproduction (R_0) tested under different Cd concentrations. An asterisk indicates a significant effect.

Long-term exposure (µg Cd/L)	Tested Cd Concentration (µg Cd/L)	Factors	Df	F	p-value
0	0	Intercept	1	222.06	0.00*
		Aquaria	2	1.29	0.31
		Clone(aquaria)	12	1.41	0.22
	2.02	Intercept	1	1198.26	0.00*
		Aquaria	2	0.01	0.99
		Clone(aquaria)	12	1.12	0.39
	4.7	Intercept	1	344.60	0.00*
		Aquaria	2	1.48	0.26
		Clone(aquaria)	12	0.54	0.87
	9.19	Intercept	1	266.30	0.00*
		Aquaria	2	5.34	0.02*
		Clone(aquaria)	12	0.63	0.80
	17.89	Intercept	1	64.76	0.00*
		Aquaria	2	3.37	0.06
		Clone(aquaria)	12	1.29	0.28
2.11	0	Intercept	1	301.73	0.00*
		Aquaria	2	0.059	0.94
		Clone(aquaria)	11	0.81	0.63
	2.02	Intercept	1	267.68	0.00*
		Aquaria	2	0.85	0.45
		Clone(aquaria)	11	2.09	0.06
4.7	0	Intercept	1	95.32	0.00*
		Aquaria	2	1.51	0.26
		Clone(aquaria)	11	6.59	0.00*
	4.7	Intercept	1	35.07	0.00*
		Aquaria	2	0.45	0.64
		Clone(aquaria)	11	3.61	0.01*
9.21	0	Intercept	1	1411.53	0.00*
		Aquaria	2	0.87	0.44
		Clone(aquaria)	11	0.77	0.66
	9.19	Intercept	1	108.14	0.00*
		Aquaria	2	0.41	0.67
		Clone(aquaria)	11	0.61	0.81
20.77	0	Intercept	1	225.99	0.00*
		Aquaria	2	0.79	0.47
		Clone(aquaria)	11	39.30	0.00*
	17.9	Intercept	1	86.69	0.00*
		Aquaria	2	0.39	0.68
		Clone(aquaria)	11	2.33	0.03*

Table 6.2: General Linear Model for effects in the Cd-evolved population with aquria (fixed factor) and clone effect nested in aquaria (random factor) on population growth rate (r_m) tested under different Cd concentrations. An asterisk indicates a significant effect.

Long-term exposure (µg Cd/L)	Tested Cd Concentration (µg Cd/L)	Factors	Df	F	p-value
0	0	Intercept	1	199.71	0.00*
		Aquaria	2	1.10	0.36
		Clone(aquaria)	12	1.64	0.14
	2.02	Intercept	1	1114.11	0.00*
		Aquaria	2	0.34	0.71
		Clone(aquaria)	12	0.89	0.56
	4.7	Intercept	1	405.03	0.00*
		Aquaria	2	2.60	0.11
		Clone(aquaria)	12	0.46	0.92
	9.19	Intercept	1	17.47	0.00*
		Aquaria	2	3.09	0.06
		Clone(aquaria)	12	1.79	0.10
	17.89	Intercept	1	87.21	0.00*
		Aquaria	2	3.69	0.04*
		Clone(aquaria)	12	1.80	0.10
2.11	0	Intercept	1	635.59	0.00*
		Aquaria	2	0.60	0.55
		Clone(aquaria)	11	1.19	0.34
	2.02	Intercept	1	258.94	0.00*
		Aquaria	2	0.077	0.92
		Clone(aquaria)	11	1.11	0.39
4.7	0	Intercept	1	90.15	0.00*
		Aquaria	2	1.328	0.29
		Clone(aquaria)	11	6.41	0.00*
	4.7	Intercept	1	35.99	0.00*
		Aquaria	2	0.55	0.58
		Clone(aquaria)	11	3.52	0.00*
9.21	0	Intercept	1	1140.64	0.00*
		Aquaria	2	0.41	0.67
		Clone(aquaria)	11	0.87	0.58
	9.19	Intercept	1	102.86	0.00*
		Aquaria	2	0.49	0.61
		Clone(aquaria)	11	0.64	0.78
20.77	0	Intercept	1	249.71	0.00*
		Aquaria	2	1.46	0.26
		Clone(aquaria)	11	30.92	0.00*
	17.9	Intercept	1	63.13	0.00*
		Aquaria	2	0.49	0.61
		Clone(aquaria)	11	2.85	0.01*

General Linear Model analysis indicate no long-term Cd exposure effect on total reproduction (R_0) and population growth rate (r_m) in comparison with long-term control exposed populations (Table 6.4 and Table 6.5). Individual values were log(x+1) transformed.

A post-hoc Duncan test indicated a significant difference in fitness between long-term 0 µg Cd/L exposed population and long-term 20.77 µg Cd/L exposed population (long-term exposure effect).

Table 6.4: General Linear Model for effects of aquaria netsted in long-term exposure (random factor) for clone effect nested in aquaria (random factor) and for evolved population (fixed factor) on total reproduction (R_0) tested under different Cd concentrations. Long-term exposures are the long-term Cd exposures and the long-term 0 µg Cd/L exposure. An asterisk (*) indicates a significant effect. ** indicates a post-hoc Duncan significant effect.

Long-term exposure (µg Cd/L)	Tested Cd Concentration (µg Cd/L)	Factors	Df	F	p-value
0 and 2.11	2.02	Intercept	1	1527.69	0.00*
		Aquaria (long-term exposure)	4	0.63	0.64
		Clone(aquaria(long-term exposure))	24	1.69	0.05
		Long-term exposure	1	5.11	0.07
0 and 4.7	4.7	Intercept	1	264.52	0.00*
		Aquaria (long-term exposure)	4	0.61	0.65
		Clone(aquaria(long-term exposure))	24	1.79	0.04*
		Long-term exposure	1	3.22	0.14
0 and 9.21	9.19	Intercept	1	157.26	0.00*
		Aquaria (long-term exposure)	4	1.88	0.14
		Clone(aquaria(long-term exposure))	24	0.81	0.70
		Long-term exposure	1	0.11	0.75
0 and 20.77	17.89	Intercept	1	91.70	0.00*
		Aquaria (long-term exposure)	4	1.65	0.19
		Clone(aquaria(long-term exposure))	24	1.74	0.04*
		Long-term exposure	1	2.28	0.20**

Table 6.5: General Linear Model for effects of aquaria netsted in long-term exposure (random factor) for clone effect nested in aquaria (random factor) and for evolved population (fixed factor) on population growth rate (r_m) tested under different Cd concentrations. Long-term exposures are the long-term Cd exposures and the long-term 0 µg Cd/L exposure. An asterisk (*) indicates a significant effect. ** indicates a post-hoc Duncan signfificant effect.

Long-term exposure (µg Cd/L)	Tested Cd Concentration (µg Cd/L)	Factors	Df	F	p-value
0 and 2.11	2.02	Intercept	1	3207.33	0.00*
		Aquaria (long-term exposure)	4	0.46	0.76
		Clone(aquaria(long-term exposure))	24	1.05	0.42
		Long-term exposure	1	2.62	0.16
0 and 4.7	4.7	Intercept	1	204.65	0.00*
		Aquaria (long-term exposure)	4	0.88	0.48
		Clone(aquaria(long-term exposure))	24	1.60	0.08
		Long-term exposure	1	4.17	0.10
0 and 9.21	9.19	Intercept	1	13.30	0.02*
		Aquaria (long-term exposure)	4	1.70	0.18
		Clone(aquaria(long-term exposure))	24	1.78	0.03*
		Long-term exposure	1	1.26	0.32
0 and 20.77	17.89	Intercept	1	112.60	0.00*
		Aquaria (long-term exposure)	4	1.19	0.33
		Clone(aquaria(long-term exposure))	24	1.99	0.02*
		Long-term exposure	1	2.07	0.22**

3.3. Ephippia production

Total ephippia production is presented in Figure 6.5. Results of ephippia production per aquarium is presented in Table S6.40. An one-way ANOVA indicated a marginally significant Cd effect (p=0.05) (Table 6.6). A post-hoc Duncan test (Table 6.6) indicated a significant difference between the long-term control exposures and exposures to 4.7 µg Cd/L and 9.21 µg Cd/L.

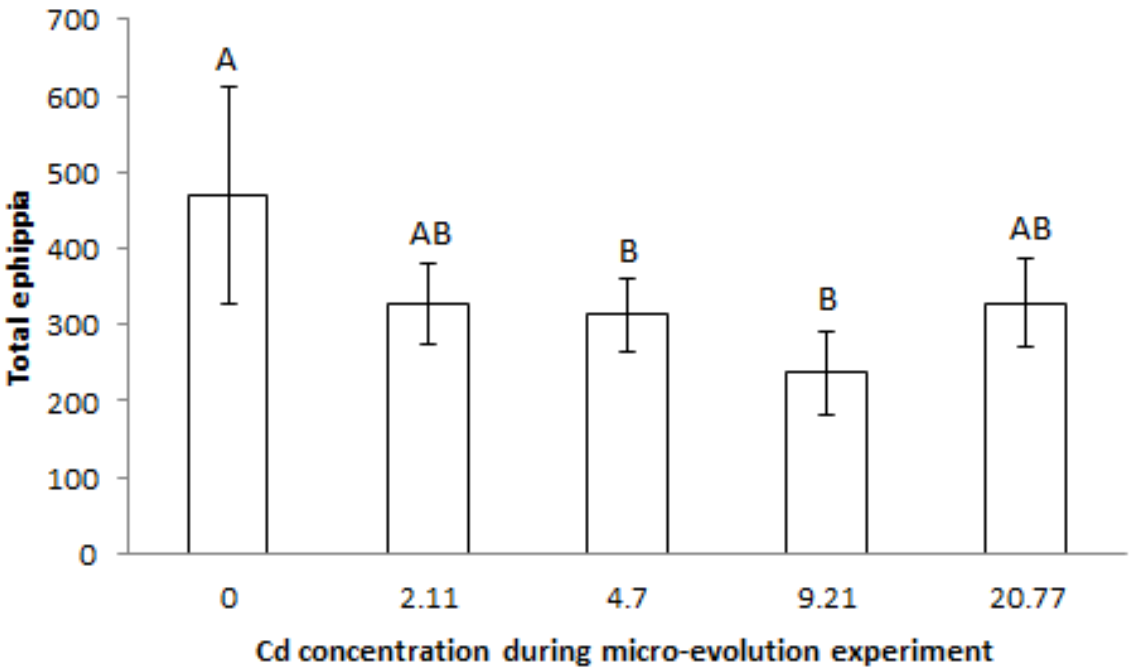

Figure 6.5: Total ephippia production during the 203-day micro-evolution experiment. Values present average ephippia production. Error bars present standard deviation.

Table 6.6: Significant differences in total ephippia production between different long-term Cd exposed populations by post-hoc Duncan test. An asterisk indicates a significant effect between the long-term Cd exposed populations.

Long-term Cd exposure	2.11	4.7	9.21	20.77
0	0.06	0.04*	0.00*	0.05
2.11		0.83	0.22	0.98
4.7			0.27	0.83
9.21				0.22

3.4. Density of the *Daphnia magna* populations during micro-evolutionary experiment

Density of *Daphnia magna* population during the micro-evolutionary experiment is presented in Figure 6.6. Results of Kruskall-wallis test, indicated significant differences between evolving *D. magna* populations at several time-points (Table S.6.41 – Table S6.42).

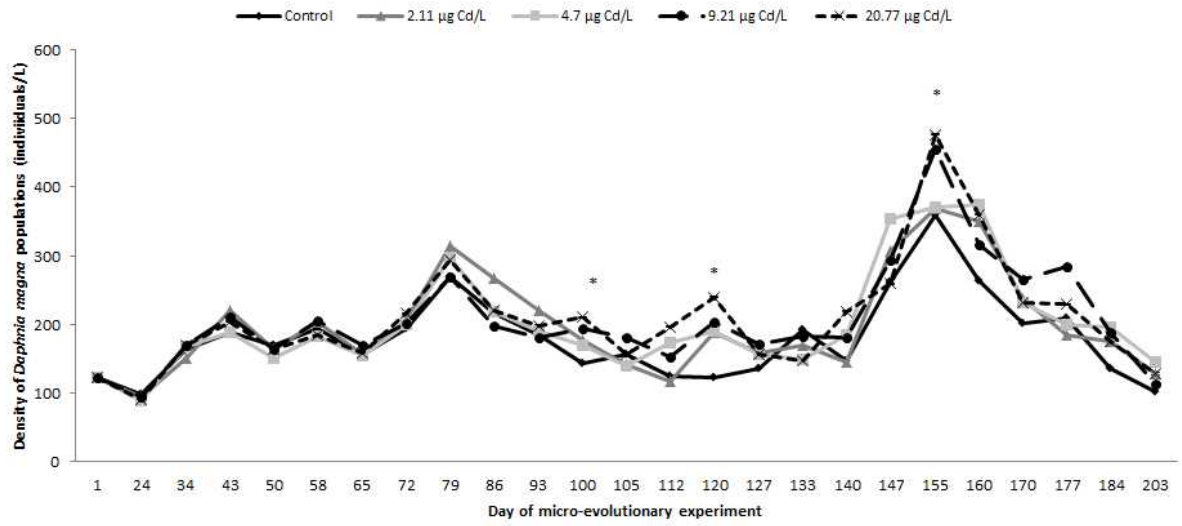

Figure 6.6: Mean density of the 5 *Daphnia magna* populations (individuals/L) during micro-evolutionary experiment. An asterisk indicates significant long-term Cd exposure effect effect.

4. Discussion

The first aim of this study was to compare fitness between the populations (all populations originated from the same Blankaart population) exposed to different long-term Cd concentrations (between 0 and 20.8 µg Cd/L) and the start population (= population kept under lab conditions). The higher fitness (total reproduction and population growth rate) (Figure 6.3 and Figure 6.4) under 17.83 µg Cd/L observed in the 20.8 µg Cd/L long-term exposed population compared to the start and the long-term control exposed population illustrates the occurrence of a change in the clonal lineages in this long-term Cd exposed population. However, a General Linear Model analysis indicates no long-term exposure effect on fitness (Table 6.5 and Table 6.6). A post-hoc Duncan test indicated that some aquaria in the long-term control exposed population had no significantly lower fitness (log(X+1) transformed) compared to the long-term 20.8 µg Cd/L exposed aquaria (Figure 6.7 and Figure 6.8).

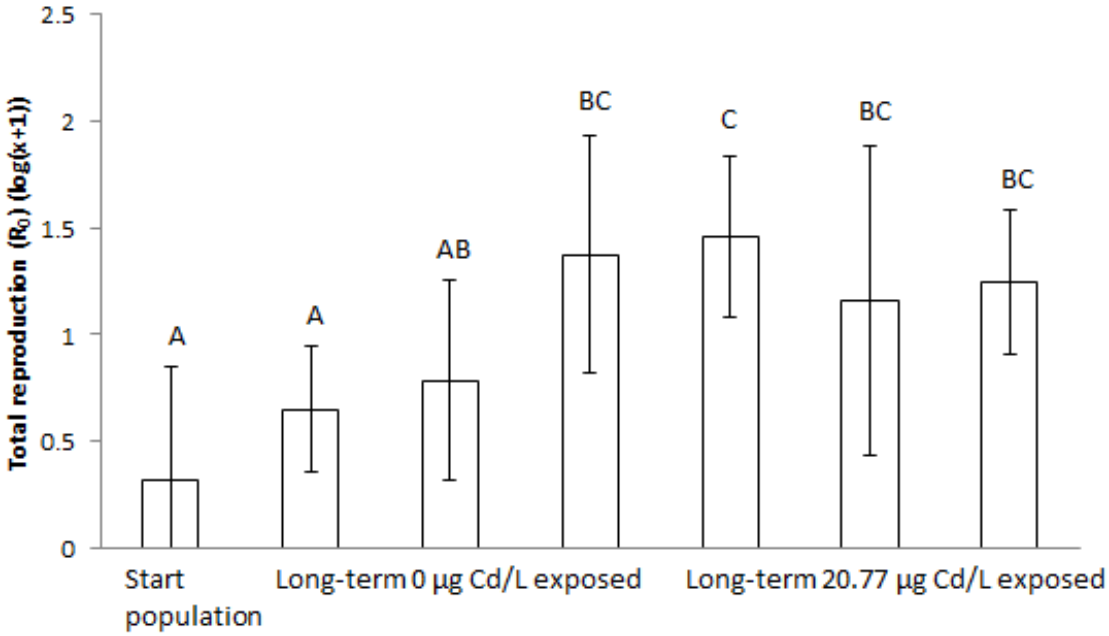

Figure 6.7: Total reproduction (log(x+1) transformed) of the different aquaria of the start population, long-term 0 µg Cd/L exposed population and long-term 20.77 µg Cd/L exposed population tested under 17.9 µg Cd/L. Aquaria that don't bear the same letter are significantly different from each other. Error bars present standard deviation.

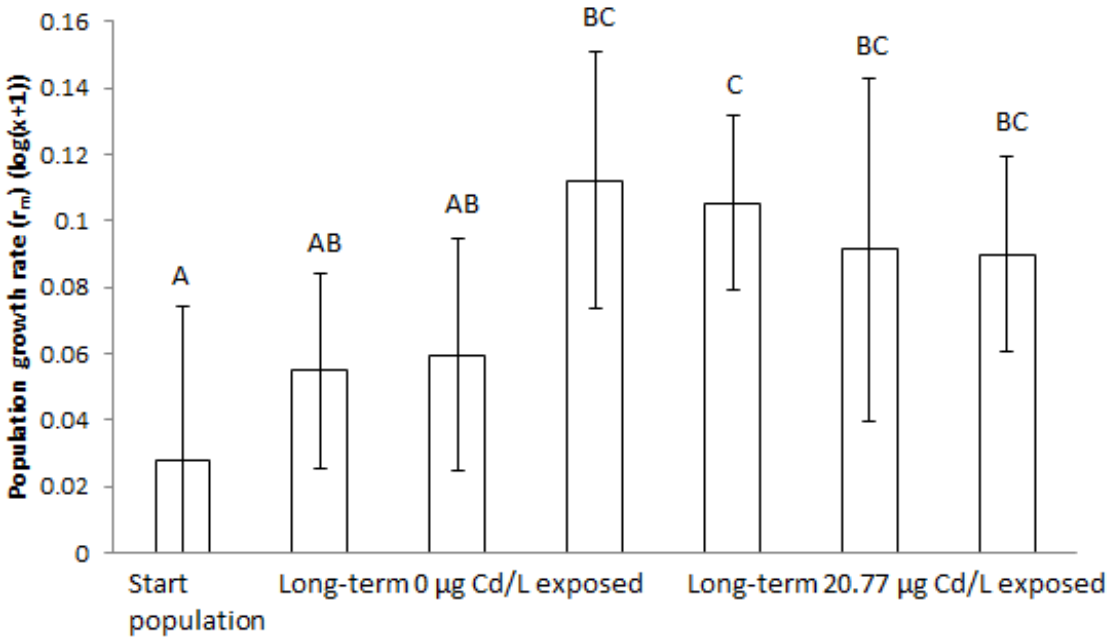

Figure 6.8: Population growth rate (log(x+1) transformed) of the different aquaria of the start population, of the long-term 0 µg Cd/L exposed population and of the long-term 20.77 µg Cd/L exposed population under 17.9 µg Cd/L. Aquaria that don't bear the same letter are significantly different from each other. Error bars present standard deviation.

A possible explanation for the found differences within the long-term control exposed population could be genetic drift, as genetic variation tends to decrease over time due to natural selection and genetic drift (Vanoverbeke et al., 2010). Ward and Robinson (2005) also found genetic drift in the control population, as the EC_{50} over the eight generations fluctuated between 61 µg Cd/L and 180 µg Cd/L. However, results of AFLP analysis indicated that selection for Cd resistance resulted in a change in genetic architecture of the Cd adapted population that could not be explained by genetic drift as found in the control populations (Ward and Robinson, 2005). A comparison of neutral markers (Fst) and quantitative trait (Qst) of the two long-term Cd exposed populations (0 and 17.8 µg Cd/L) and between the aquaria could distinguish the drift and selection effect. If the quantitative trait is under selection, then population differentiation will be more pronounced than it is for the neutral marker. In absence of selection, Fst and Qst should be similar (Klerks et al., 2011).

Increased population fitness was reported in Lopes et al. (2005, 2004, 2006), where a higher tolerance was found for the populations originating from contaminated habitats in comparison with reference populations. Agra et al. (2010) also found a higher tolerance to Cu and Zn of the populations originated from the impacted site compared to the reference site. Yet, in the studies of Lopes et al. (2004, 2005, 2006) and Agra et al. (2010), it is not possible to fully exclude that other habitat characteristics, other than the toxicant, may have influenced the evolved tolerance. Similar results were found in Xie and Klerks (2003), where a rapid response to selection for Cd tolerance was observed in the least Killifish. Ward and Robinson (2005) also observed an increased tolerance for Cd in a *D. magna* population during a selection experiment. This latter experiment used an artificial population of only 8

clones, where acclimation effects could not be ruled out. Lopes et al. (2009) found that AMD (= acid mine drainage) led to a dramatic change in clone frequencies, where the most tolerant clone for AMD dominated the population (although only 5 clones were used in this microcosm experiment). It has to be noted that the results of this experiment; the experiment of Ward and Robinson (2005) and Lopes et al. (2009) are based on a clonal approach (ephippia were removed during this experiment). Competition among clones during asexual reproduction may erode clonal diversity, decreasing the number of clones in the population or altering the relative frequency of clonal lineages (Vanoverbeke et al., 2007). For example, Ward and Robinson (2005) observed a loss of genetic variation in the (asexually) Cd-adapted population, i.e. an average 68% reduction in diversity after 8 generations under Cd pressure (Cd concentration of 61 µg Cd/L). While asexual reproduction is supposed to assure the maintenance of a superior genotype in an environment, sexual reproduction is considered to provide the basis for rapid evolution (Doroszuk et al., 2006). Yet, several studies indicated that asexual organisms were able to respond rapidly to strong selection (Ward and Robinson, 2005; Brausch and Smith, 2009; Doroszuk et al., 2006). The study of Chaumot et al. (2009) suggested, because of the lack of additive genetic variance, that the apparent adaptation found in the study of Lopes et al. (2004, 2005) (i.e. increased tolerance in contaminated populations) would disappear at each sexual reproduction event. In contrast, as indicated in chapter 4, there is an indication of additive genetic variance in the same tested Blankaart *D. magna* population under Cd stress (3.6 µg Cd/L).

There is also an indication that there was a response to selection or due to drift in the long-term control exposed population, as significant (by non parametric bootstrap resampling) higher fitness was noted in the 2.02, 4.7 and 17.83 µg Cd/L treatment in this

population compared to the start population (=original population). This indicates that temperature, food density, other non-measured parameters or a combination of these parameters may have a selection effect or drift may also have played a role (resulting in loss of clonal diversity). Ward and Robinson (2005) also observed a reduced genetic diversity (-53%) in a control population over eight generations compared to the initial population in a selection experiment. Similar results were found with the control evolution treatment in the study of Lopes et al. (2009), where there was a significant reduction of diversity in control conditions (no disturbance) and a great reduction under strong AMD exposure.

There was no significantly lower density during the micro-evolution experiment in the long-term 20.8 µg Cd/L exposed population and there was no significant difference in ephippia production (Table 6.6). Although long-term 4.7 µg Cd/L and 9.21 µg Cd/L exposed populations had a lower ephippia production compared to the long-term control exposed population. Shift in sex ratio has been found for several toxicants. Peterson et al. (2001) found more female broods in *Daphnia pulex* exposed to methoprene. More male broods were produced when egg-bearing daphnids were exposed to 20-hydroxuecdysone (Peterson et al., 2001). Dong et al. (1999) found a shift in sex determination toward males when *Daphnia pulicaria* females were exposed to atrazine (see Rodriguez et al., 2007 for review). Deng et al. (2010) found a negative correlation between the presence of *Microcystis aeruginosa* in the diet of *D. carinata* and ephippia production. In contrast a positive correlation was found for *D. pulex*.

The observed higher population fitness (although an aquaria effect was found) under 17.9 µg Cd/L from the long-term 20.77 µg Cd/L exposure is believed to be advantageous for populations inhabiting contaminated ecosystems, however, costs are often associated (i.e.

cost of tolerance) (Brausch and Smith, 2009). In this study no such cost was observed (Figure 6.3 and Figure 6.4). Results of chapter 2 and chapter 3 and other studies (Brausch and Smith, 2009; Miyo et al., 2000) indicate no such cost, although some studies have demonstrated the opposite effect (Shirley and Sibly, 1999; Postma et al., 1995a; Levinton et al., 2003). Although, results of Ward and Robinson (2005) indicated no effect on fecundity, Cd-adapted daphnids were smaller and showed greater sensitivity to phenol, however not to lead. Lopes et al. (2009) also found that the tolerant clones for lethal levels of AMD were also Cu tolerant. However, the most AMD resistant clones were the most sensitive to Cd. Development of increased tolerance and its associated consequences (cost of tolerance) can have implication on ecological risk assessment (Klerks and Weis, 1987; Brausch and Smith, 2009).

5. Conclusion

The long-term 20.8 µgCd/L exposure of a *Daphnia magna* population in a micro-evolutionary experiment resulted in a population that had a higher fitness in 17.9 µg Cd/L compared to a long-term control exposed population and start population. However, genetic drift in the aquaria could not be excluded. It appears that this long-term Cd exposed population had no associated costs with this increased fitness, i.e. no cost of adaptation. Although change in fitness trade-offs and tolerance for second stressors were not monitored, which could influence the outcome of our study. As ephippia during this experiment were removed, this gives an indication of clonal selection, although subsequent experiments should determine the inheritance for Cd tolerance to the next generations.

Chapter 7: Putting results in perspective: Conclusions

Conventional risk assessment of chemicals is based on the mean population response of selected life-history traits to chemical exposure and does not take into account the genetic variability of that response (Forbes and Forbes 1994; Barata et al. 2000b). As a consequence, it does also not account for potential micro-evolutionary responses to chemical exposure such as directional selection in natural populations. Yet, while directional selection may lead to genetic adaptation and persistence of the population under the chemical exposure, it may also lead to a reduction of genetic diversity, with potentially adverse long-term outcomes (i.e. cost of adaptation, see also chapter 2 - chapter 3 - chapter 6). Thus, until the relation between selection, potential for adaptation, reduction of genetic diversity and cost of adaptation is better understood, it may be of interest to environmental regulators to know if such responses are likely (or unlikely) to occur at concentrations that are considered 'safe' for the environment, according to the existing risk assessment procedures. This question is worked out in some more detail below. As there are considerable differences in the water hardness in the studies cited below, and as hardness is known to affect Cd toxicity, all Cd concentrations mentioned below have been corrected to a reference hardness of 50 mg $CaCO_3$/L, by multiplying the Cd concentration with a factor of $(50/\text{hardness})^{0.7409}$ as in the Cd RAR and EQS documents (ECB 2007; EU 2008). This is the same correction as the one used by US-EPA (2001) for its chronic aquatic life criterion for Cd. The original Cd concentrations, hardness levels and corrected Cd concentrations for each of these studies are presented in Table 7.1. To discriminate with non-corrected Cd concentrations, we refer to the hardness-corrected Cd concentrations in the text below with an asterisk (*), i.e. µg Cd*/L.

Table 7.1: Summary of different studies that deal with micro-evolutionary responses to Cd in freshwater. The original Cd concentrations (NOEC values or Cd concentration at which an increased micro-evolutionary potential was observed), hardness levels and corrected Cd* concentrations (to a hardness of 50 mg CaCO3/L) for each of these studies are presented.

Experiment	Hardness $mgCaCO_3/L$	NOEC (µg Cd/L)	Hardness Corrected NOEC (µg Cd*/L)	Cd concentration (µg Cd/L)	Hardness corrected Cd concentration (µg Cd*/L)	Reference
NOEC						
NOEC of 10 *D. magna* clones used in the EU Cd risk assessment	11-300	0.16-3.2	0.07-1.84			ECB (2007)
NOEC of natural *Daphnia magna* population exposed to Cd stress as described in chapter 2	250	0.89	0.27			Chapter 2
Micro-evolutionary potential						
Increased micro-evolutionary potential of a natural *D. magna* field population	160			0.5	0.21	Barata et al. (2000b)
Increased micro-evolutionary potential of a natural *D. magna* population exposed to Cd stress as described in chapter 2	250			1.9 18.9	0.57 5.73	Chapter 2
Increased micro-evolutionary potential of natural Daphnia magna population to Cd stress and increased temperature as described in chapter 3	250			4.3	1.3	Chapter 3
Increased micro-evolutionary potential (CV_GR_0) in 45% of the tested *Daphnia magna* populations exposed to Cd stress as described in chapter 5	250			4.4	1.3	Chapter 5
Adaptive potential						
Absence of adaptive potential (no significant heritability) of lethal Cd tolerance of *Gammarus fossarum*	110			20 (absence of heritability)	6.07 (absence of heritability)	Chaumot et al. (2009)
Adaptive potential (significant heritability) of Cd on *Daphnia magna* population as described in chapter 4	250			3.8	1.15	Chapter 4
Selection response						
Selection response leading to increased fitness of the population in a selection experiment	250			20.77	6.83	Chapter 6
Increase in Cd resistance observed in an artificial population of 8 *D. magna* clones	170			61	24.6	Ward and Robinson (2005)
Observation of adaptive response of natural *Chironomus riparius* population to Cd in the field	128			13-54.4	6.57-27.11	Postma et al. (1995b) Postma et al. (1996) Groenendijk et al. (1999a,b)

In the EU risk assessment arena, the no observed effect concentration (NOEC) and the 10% effective concentration (EC_{10}) are most commonly used as inputs to the derivation of predicted no effect concentrations (PNEC) in chemicals risk assessment (ECHA 2008) or environmental quality standards (EQS; EU 2008; EU 2005). For chemicals for which chronic NOECs are available for multiple species, including Cd, the PNEC and EQS can be derived with the statistical extrapolation technique. In the EU, both the PNEC of Cd, derived in the Cd Risk Assessment Report (Cd-RAR; see ECB, 2007), and the EQS for Cd, derived in the context of the water framework directive (EU, 2005) are 0.09 µg Cd*/L. A species sensitivity distribution was fitted to hardness-corrected NOEC data for 28 freshwater species. Among those species, *D. magna* was among the most sensitive ones (with a geometric mean hardness-corrected NOEC of 0.51 µg Cd*/L). Next, the hazardous concentration for 5% of the species (HC_5) was estimated at 0.18 µg Cd*/L and an assessment factor of 2 (to cover 'residual uncertainty') was applied to that value to derive a PNEC = EQS = 0.09* µg Cd/L. This PNEC is below the result of the micro-evolution experiment (chapter 6), where there was an increased fitness observed at 6.30 µg Cd*/L (compared to the long-term control exposed population) exposed to 5.43 µg Cd*/L. Studies with *D. magna* populations suggest an increased micro-evolutionary potential at 0.21 µg Cd*/L (Barata et al. 2000b), 5.73 µg Cd*/L (chapter 2), 1.3 µg Cd*/L (chapter 5) or 24.6 µg Cd*/L (Ward and Robinson, 2005). The latter is based on a micro-evolution experiment with an artificially constructed population consisting of 8 *D. magna* clones. A combination with temperature increase (20°C to 24°C) suggested an increased potential for directional selection at 1.3 µg Cd*/L (chapter 3). Although for the Blankaart population (chapter 3) no significant increased micro-evolutionary potential was noted at 20°C, 45% of the tested *Daphnia magna* populations in chapter 5 had a significant increased micro-evolutionary potential (expressed as CV_G, in

terms of total reproduction) at 1.3 µg Cd*/L. The found differences among populations for the quantitative trait may have a genetic basis or is due to random genetic drift rather than selection, although in this study no differentiation between natural selection or genetic drift between populations could be distinguished. Comparsion of Qst and Fst values could give more indication and insight between natural selection and genetic drift. The logic behind is that Qst is a measurement for a quantitative trait, while Fst is a measure for a neutral genetic marker. If Qst is under selection, the differentiation between populations for that trait will be more pronounced than it is for a neutral marker. If natural selection is absent, Fst and Qst will be similar (Klerks et al., 2011). The use of neutral markers can be a powerful tool to investigate the effects of contaminants (or stressors in general) on genetic diversity. The backside is that a lot of data are required. Carvajal-Rodriquez et al. (2005) found that 10-20 neutral genetic markers are needed to provide the same information as a single quantitative genetic trait. The use of these neutral genetic markers is been proven in a study of Coors et al. (2009). In this study all individuals from 10 *D. magna* populations were genotyped at four polymorphic allozyme loci. The authors could find a relationship between genetic diversity and land-use intensity which suggests genetic erosion correlated with anthropogenetic pollution. However, population toxicant susceptibility was not correlated with population genetic diversity, which indicates that genetic diversity measured by neutral markers does not itself promote tolerance to toxicants (Coors et al., 2009). Lind and Grahn (2011) investigated the genetic difference between populations of *G. aculeatus* from reference and paper-mill contaminated sites. Genetic variability was determined by Amplified Fragment Length Polymorphism (AFLP). The genetic composition of these multiple populations have responded to the directional selection pressure from the effluents of paper pulp mills in the Baltic Sea.

The H^2 (chapter 2 - chapter 3 - chapter 4 - chapter 5), also called "the degree of genetic determination" (Klerks et al., 2011) might be useful as a predictor of adaptive potential for asexual reproducing organisms like *Daphnia magna*. The narrow sense heritability (h^2) is often considered as the best predictor of a population's potential to respond to selection (Klerks et al., 2011) during sexual events. The narrow sense heritability (h^2), i.e. the ratio of the additive genetic variance divided by the total phenotypic variance (Falconer and Mackay, 1998), of tolerance traits is considered a prerequisite for a population's ability to evolve increased tolerance by natural selection. The magnitude of h^2 may give a good idea about the possible rate of adaptation (Klerks et al., 2011). It should be noted that H^2 and h^2 provide predictions of the absolute change of a trait following selections, while the total genetic coefficient of variation (CV_G) and the additive coefficient of variation (CV_A) would provide a better prediction of the relative change (Klerks et al., 2011; Houle, 1992). As we had no specific a priori interest in either absolute or relative trait changes, we have considered both heritabilities and genetic coefficients of variation. Heritability estimates (H^2) may not be the most relevant predictor of selection response for sexually reproducing organisms. Only additive genetic effects are inherited by the next generation in case of sexual reproduction (Lynch and Walsch, 1998). Therefore, if no additive genetic variation of chemical tolerance existed, only a "transitory state of adaptation" could be reached by means of clonal selection during the period of asexual reproduction in *Daphnia* populations (Chaumot et al., 2009) which could "disappear" again with every sexual reproduction event. The existence of significant additive genetic variation (i.e. significant h^2) of fitness is necessary for the persistence of any fitness increase in the population that may have built up through natural selection of fitter clones during the preceding of asexual reproduction. In chapter 4, we found significant h^2 and CV_A under increased temperature

stress (24°C versus 20°C) and 5 µg Cd/L. The genetically determined ability to maintain higher fitness under Cd stress is partially heritabile to sexually produced offspring, but only at 24°C and not at 20°C. This finding indicates that the adaptive potential of a natural *D. magna* population to chemical stress may be dependent not only on the presence of the chemical stress, but on other environmental variables as well (e.g. temperature). This was in contrast with Chaumot et al. (2009) who indicated that there was no potential for directional selection in a natural *Gammarus fossarum* population at 11.2 µg Cd*/L, due to the absence of heritable genetic variation of Cd tolerance. Chaumot et al. (2009) hypothesized that "a wide-spread weakness of standing additive genetic variation of chemical tolerance in natural populations". This means that genetic adaptation to contaminant exposure would be more likely to take place through fixation of rare (beneficial) alleles rather than through natural selection acting on standing variation. Our findings suggests that at least *D. magna* populations would be able to acquire and maintain increased resistance to Cd through natural selection acting on standing genetic variation without the need of beneficial mutation followed by their fixations. However, heritability estimates may have limited applicability (Klerks et al., 2011) as this estimate is only valid for the trait, species and population for which heritability is determined. Estimations depend also on environmental conditions, which determine the environmental variance (as part of the total phenotypic variance). So stressful environments influence the heritability estimate (Bubliy and Loeschche, 2002). The heritability of a single trait may not provide sufficient information to predict the trait's responses to selection and does not provide insight into other traits that might change simultaneously (Klerks et al., 2011). It has been shown that evolution can be constrained by genetic correlations among fitness-related traits (Reznick et al., 2000), also commonly referred to as trade-offs. If the presence, for example, of a toxicant result in a

selection pressure for increased resistance to that contaminant and this trait has negative genetic correlation tied to fitness, then the response to selection will be slowed down by that negative genetic correlation. As described in chapter 2, chapter 3 and chapter 6, no cost of tolerance was observed under Cd stress (Cd range between 0 - 6.83 µg Cd*/L). As described in chapter 3, temperature increase (20°C vs 24°C) and Cd (1.3 µg Cd*/L) may affect the between-trait correlations in a *D. magna* populations although more statistical evidence was needed. A meta-analysis study of Agrawal and Stinchcombe (2009) found no strong evidence for such constraints. Besides genetic correlations between traits, a contaminant may effect a selective pressure on multiple traits and these traits are likely fully independent. A multivariate approach in which the equivalence to the additive genetic index becomes the additive genetic variance-covariance matrix, or G-matrix. This approach could be useful for addressing micro-evolutionary changes related to environmental contamination (Klerks et al., 2011).

In addition to the comparison of PNEC and EQS with concentrations at which quantitative genetics studies (i.e. determining genetic variation and heritability of fitness) and micro-evolution experiments have been conducted, it is also instructive to perform a comparison of PNEC and EQS with concentrations at which micro-evolutionary, adaptive responses have been observed in the field. Adaptation for a *C. riparius* population from a historically metal-contaminated stream, as evidenced by increased tolerance to Cd, was observed in a concentration range of 6.47 to 27.11 µg Cd*/L, which is also considerably higher (72 to 301-fold) than the PNEC and EQS of 0.09 µg Cd*/L. This increase could be considered as an ecologically positive event. On the other hand, increased tolerance of the population to a chemical stress due to directional selection of tolerant genotypes may also

have adverse long-term ecological consequences. Indeed, natural selection results by definition in a reduction of the genetic diversity (Lynch and Walsh, 1998). This in turn may lead to a decreased tolerance to other stressors (Ward and Robinson, 2005), a reduced adaptive potential towards future challenges imposed by novel stressors (Van Straalen and Timmermans, 2002) or a reduced fitness when the selective pressure is removed (e.g., after remediation of a polluted site) an observation which is commonly referred to as "cost of tolerance" (Medina et al., 2007). This phenomenon is caused by genetic between-environment correlations or between-environment trade-offs (Medina et al., 2007). As indicated in chapter 2, chapter 3 and chapter 6 those cost of tolerance could not be found. This was general found in other studies with aquatic invertebrates (Agra et al., 2010). A study of Salice et al. (2010) indicate that parasite-resistant and susceptible strains of a freshwater snail exposed to Cd had a decreased tolerance to temperature stress. This is in discrepancy with the results of chapter 2 – chapter 3 and chapter 6. Fisker et al. (2011) found that *D. octaedra* living in soils with higher copper concentrations do not carry any apparent costs of adaptation. Other studies indicate such costs. Thereby the investigated *Daphnia magna* population used in chapter 2 - chapter 3 and chapter 6 could be fraught with costs that have not been revealed in these studies. However as not dealt in chapter 2 - chapter 3 - chapter 5 - chapter 6 the underlying mechanism for the associated costs would provide insight.

In conventional ecotoxicology, monoclonal laboratory *D. magna* populations are widely used. Additionally, we determined effect concentrations (i.e. 21d-NOEC, EC_{10} and EC_{50} based on the R_0 endpoint) for 7 monoclonal populations of *D. magna* obtained from 7 different European ecotoxicology laboratories (Table S7.2 for more detailed information).

For *D. magna* the standard and therefore most commonly used endpoint is R_0, as determined in 21-day life-table tests according to OECD test guideline No. 211 (OECD 1998). Next to the NOEC and the 21d-EC_{50} (median effective concentration) is also reported here, because it can usually be estimated with more precision that the EC_{10}. The test design was the same as described in chapter 2 (see detailed information in Supplementary Material S7.1). As shown in Table 7.2, we observe a considerable inter-clonal variation of Cd toxicity (5-fold for 21-day EC_{50}). This inter-clonal variation of Cd toxicity corroborates with many other ecotoxicity studies with different *D. magna* clones and Cd. For instance, Baird et al. (1990) reported an up to 100-fold variation of acute Cd tolerance and Barata et al. (2002b) reported a 7-fold variation of the EC_{50} of Cd for feeding rate. Barata et al. (2002a) argued that every isolated population of *D. magna* may evolve different stress tolerances (including Cd tolerance) due to among-habitat differences of selective forces (not necessarily related to the stress one investigates, in this case Cd). In addition, there is also considerable inter-clonal variation of Cd tolerance within populations as shown here and elsewhere (chapter 5; Barata et al., 2000; Messiaen et al., 2010). Hence, it is not surprising that *D. magna* clones collected from different ecotoxicology laboratories from across Europe exhibit a wide range of Cd sensitivities, considering that all these clones have once been isolated from potentially very different regions and habitats. The genetic variation of chemical tolerance among clones of the same species is not unique to Cd and *Daphnia*. Other examples include the genetic variation of chronic azoxystrobine tolerance in *D. magna* (Warming et al., 2009), acute LC50s of Cd among three clones of *Potamopyrgus antipodarum* (Jensen and Forbes, 2001), and 35-day toxicity of chlorpyrifos to *Folsomia candida* (Crommentuijn et al., 1995). This suggests that the findings in the present study may have implications that go beyond just *D. magna* and Cd.

Table 7.2: Chronic toxicity data for 21-day net reproductive rate (R_0) for monoclonal *D. magna* populations from 7 different European Laboratoria. * indicates hardness corrected

Clone ID	CZ	K6	SE	DK	F	A	IRCHA type 5
21d-NOEC (µg Cd/L)	(>18.9)	1.92	(>18.9)	8.34	(3.96)	0.89	8.34
21d-NOEC (µg Cd*/L)	(>5.73)	0.58	(>5.73)	2.53	(1.20)	0.27	2.53
% effect at NOEC	(56%)	16%	(33%)	1%	(54%)	12%	12%
21d-EC_{10} (µg Cd/L)	-	1.75 (0.81-4.41)	10.4 (3.0-35.3)	-	1.49 (0.68-3.28)	0.31 (0.07-1.27)	11.8 (2.3-60.8)
21d-EC_{10} (µg Cd*/L)	-	0.53 (0.24-1.33)	3.16 (0.9-10.71)		0.45 (0.21-0.99)	0.09 (0.02-0.39)	3.58 (0.70-18.45)
21d-EC_{50} (µg Cd/L)	>8.39	5.63 (4.06-7.80)	20.1 (14.5-27.7)	- (8.34-18.9)	4.66 (3.33-6.52)	3.80 (2.17-6.67)	17.7 (13.3-23.6)
21d-EC_{50} (µg Cd*/L)	>5.73	1.70 (1.23-2.37)	6.09 (4.4-8.4)	- (2.53-5.74)	1.45 (1.01-1.98)	1.15 (0.66-2.02)	5.73 (2.03-7.16)
% effect at 18.9 µg/L (5.73 µg Cd*/L)	56%	92%	33%	87%	91%	75%	62%

[a] NOEC = no observed effect concentration, EC10 and EC50 are 10% and 50% effective concentrations; NOECs were only considered reliable if a less than 20% effect was observed. The % effect was calculated by { R_0(control)-R_0(Cd) } / R_0(control) and represents the % reduction of the mean R_0 at a Cd treatment compared to the control treatment. Unreliable NOECs are reported between parentheses and are not considered in the discussion.

While we have so far focused on the genetic variation of the response of the natural population's fitness to Cd, risk assessment is so far still exclusively using the mean population response of selected life-history traits (Forbes and Depledge 1996; Barata et al. 2000a). Table 7.1 reports the population's mean responses for total reproduction and population growth rate (i.e. the mean of the different clone's responses) to increasing Cd concentrations from the study in chapter 2. The population mean of total reproduction (R_0) at 1.9 µg Cd/L was significantly ($p<0.05$) lower than in the control. Hence, the 21d-NOEC for total reproduction for the natural population was 0.89 µg Cd/L (i.e. the no observed effect concentration for a single-generation exposure). The NOEC for population growth rate (r_m) for the natural population was also 0.89 µg Cd/L. Two considerations are of interest. First, this natural population's NOECs are at the lower range of the NOECs for the same endpoint observed with the laboratory clones. This indicates that using a single NOEC of a randomly selected laboratory clone of *D. magna* for risk assessment may not be protective for a randomly selected natural population. This is one of the many uncertainties associated with lab-to-field extrapolation which is currently supposed to be covered by the current (largely arbitrary) assessment factors applied in conventional risk assessment (Forbes and Calow, 1999). A second observation of interest relates to the toxicity test results of two of the laboratory clones. Clone A, currently held in a Portuguese laboratory (see Table S7.2) and frequently used in other papers and studies (Barata et al., 2002), was originally established from individuals of the monoclonal population IRCHA type 5, currently held in a French laboratory. Thus, one would expect similar sensitivities to Cd. However, in contrast, amongst all laboratory clones investigated in the present study, clone A and clone IRCHA type 5 were the clones with the highest and lowest sensitivity to Cd, respectively. The 21-d NOECs were 0.89 and 8.34 µg Cd/L (A and IRCHA type 5, respectively), the 21d-EC_{10} were 0.31 and 11.8 µg

Cd/L, and the 21d-EC_{50} were 3.8 and 17.7 µg Cd/L. Since both clones were cultured for several generations under identical conditions in our laboratory prior to testing, it is likely that over the course of several years genetic differentiation between the two 'monoclonal' laboratory cultures, which once represented 'the same clone', may have played a role. Baird (1992) hypothesized that mutations in a monoclonal *D. magna* population could lead to an altered genetic constitution of that population. As such, genetic differentiation between two monoclonal cultures established from the same clone may arise. When these mutations occur at genes that are involved in chemical tolerance this may randomly lead to a differentiation of chemical tolerance between the two populations. Another possible explanation is epigenetic inheritance. Vandegehuchte et al. (2009) recently indicated that environmentally-induced epigenetic effects (i.e. changes in DNA methylation) can occur in a monoclonal *D. magna* culture and that these effects can be inherited, i.e. maternally transferred (Vandegehuchte et al., 2010). Therefore, if culture conditions of the same clone among two laboratories are different, epigenetic processes could theoretically also lead to a differentiation of the epigenome among the two cultures, with potential implications for chemical tolerance.

Returning to our main research question, we compared the conventional effect concentrations reported above (Table 7.2) with the concentration of Cd where we have seen an increased potential for a micro-evolutionary response. In chapter 2 we found a higher micro-evolutionary potential at 0.57 µg Cd*/L (based only on $CV_G(r_m)$) and at 5.73 µg Cd*/L (based on $CV_G(R_0)$ and $H^2(r_m)$). All 21d-NOEC and 21d-EC_{10} values of the laboratory clones are lower (between 1.6 and 61 fold) than 5.73 µg Cd*/L (chapter 2). The range of 21d-EC_{50}s encompasses 5.73 µg Cd*/L, suggesting that an increased micro-evolutionary potential in a

natural population may be close to 50% reproductive inhibition (or higher) in standard tests with laboratory clones. This finding is supported by the range of reproductive inhibitory effects in the same laboratory clones at 5.73 µg Cd*/L, which was between 33% and 92% (Table 7.2). Incorporating the results of chapter 3, chapter 4 and chapter 5, we indicated that there was a significantly increased micro-evolutionary potential at 1.3 µg Cd*/L (at 24°C for the Blankaart population (chapter 3 and chapter 4) and 45% of the tested populations at 20°C in chapter 5), which suggests that for some populations an increased micro-evolutionary potential may occur in the range of conventional NOEC or EC_{10} values. This increased micro-evolutionary potential may lead to shifts in genotype frequencies (Van Sraalen and Timmermans, 2002). Based on the results of a semi-natural micro-evolution experiment (chapter 6), we have only found evidence of a micro-evolutionary response of the *D. magna* population from the Kasteelvijver pond at 5.73 µg Cd*/L (hardness corrected). As mentioned above, this is higher than all conventional 21d-NOECs and 21d-EC_{10}s (0.09 – 5.73 µg Cd*/L) for the laboratory *D. magna* clones in the present study, and also higher than all chronic NOECs that were accepted in the Cd RAR and EQS document, i.e. 0.07 - 1.84 µg Cd*/L. Thus, based on evidence of the micro-evolutionary experiment for the Kasteelvijver pond *D. magna* population, no increased micro-evolutionary potential is expected at conventionally derived NOECs.

In the EU, when chronic NOECs for a chemical are available for three trophic levels (typically an alga, a fish and *Daphnia* sp.), the lowest NOEC (most sensitive species) is conventionally divided by an assessment factor of 10 to obtain the PNEC or EQS. These concentrations are considered to have 'no effects' on freshwater populations, communities and ecosystems. For many chemicals, *D. magna* is the most sensitive species tested

(Wogram and Lies 2001; Von der Ohe and Lies, 2004) and for many chemicals a NOEC will only be available for a single *D. magna* clone. The data with Cd cited above show that a single NOEC from a randomly selected *D. magna* clone can be more than 10-fold higher than the concentration at which an increased micro-evolutionary potential is observed (i.e. 0.21 µg Cd*/L) (Barata et al., 2002b). In such a case, a micro-evolutionary response may theoretically be possible, at a conventionally derived PNEC or EQS value. When considering all laboratory clone NOECs cited above (or EC_{10}s if reliable NOECs were not available) (both present study and Cd RAR), 3 out of 17 (i.e. 18%) were more than 10-fold higher than 0.21 µg Cd*/L. However, none of the labo NOECs is 10-fold higher than the concentrations with true micro-evolutionary responses (Table 7.1 and Table 7.2) (except for the population of Barata et al., 2000b). Nonetheless, the previous thought exercise calls for additional research that compares (ranges of) *conventional D. magna* NOECs with concentrations of chemicals that invoke increased micro-evolutionary potential and responses in natural *D. magna* populations (compared to control conditions).

In conclusion, *Daphnia magna* has been a model organism in a broad range of studies: ecotoxicology, ecology, evolutionary biology. The interplay between these kinds of research would broaden the knowledge of the underlying mechanisms of the potential for adaptation to stressors. Therefore, it would be recommended for further studies to use genome scan analysis to identify genome regions that are under selection. If genes, identified through genome scan, are genes of large effect, these genes can be used as a proxy for traits. This would reduce the time effort of quantifying genetic variability for ecologically relevant traits. Additionally, this genome wide analysis involving a large number

of markers allows a more detailed analysis of genetic structure of the population(s), differentiating between neutral markers and markers under selection.

All the evidence so far indicates that an increased micro-evolutionary response to Cd exposure, which may lead to shifts in genotype frequencies, may only occur above the conventionally derived PNEC and HC_5 of Cd. However, given the large differences between populations of the same species (i.e. the Kasteelvijver pond population, the populations tested chapter 5 and the Spanish *D. magna* population of Barata et al. (2002b)) and between different species (i.e. *D. magna* vs. *G. fossarum* vs. *C. riparius*), it is recommended to broaden the knowledge to more *D. magna* populations and to additional species as well. As field populations are influenced by a broad range of stressors and our results indicate that temperature increase affects the outcome of our studies, it would be recommended to assess the impacts of multiple stressors and/or impacts of global change. As there were comparable results found in chapter 6 and chapter 2, micro-evolutionary potential derived from short term tests (i.e. one generation) could be used instead of long-term studies (cfr. chapter 6). So it would be interesting to determine the micro-evolutionary potential of different populations at derived PNEC and/or EQS of other chemical substances. In addition, the fact that it is theoretically possible that PNEC and EQS derived from a single *D. magna* NOEC may be higher than the concentration at which micro-evolutionary effects may be possible, calls to extend this kind of research to multiple chemicals/stressors.

References

Agra, A.R., Guilhermino, L., Soares, A.M.V.M., Barata, C.. 2010. Genetic costs of tolerance to metals in Daphnia longispina populations historically exposed to a copper mine drainage. Environmental Toxicology and Chemistry, 29(4): 939-946.

Agrawal, A.A., Stinchcombe, R.. 2009. How much do genetic covariances alter the rate of adaptation? Proceedings of the Royal Science series B 276: 1183-1191.

Arbaciauskas, K., Lampert, W.. 2003. Seasonal adaptation of ex-ephippia and parthenogenetic offspring of *Daphnia magna*: differences in life history and physiology. Functional ecology, 17: 431-437.

Baird, D.J., Barata, C.. 1998. Genetic variation in the response of *Daphnia* to toxic substances: implications for risk assessment. In ForbesVE, ed, *Genetics and Ecotoxicology.* Taylor & Francis, Philadelphia, PA, USA, pp 207–221

Baird, D.J.. 1992. Predicting population response to pollutants: in praise of clones A comment on Forbes and Depledge. Functional Ecology, 6(5): 616-617.

Barata C., Baird D.J., Amat F., Soares A.M.V.M.. 2000b. Comparing population responses to contaminants between laboratory and field: an approach using *Daphnia magna* ephippial egg banks. Functional Ecology 14(4), 513-523.

Barata, C., Baird, D.J., Minarro, A., Soares, A.M.V.M.. 2000a. Do genotype responses always converge from lethal to nonlethal toxicant exposure levels? Hypothesis tested using clones of *Daphnia magna* strauss. Environmental Toxicology and Chemistry, 19(9): 2314-2322.

Barata, C., Baird, D.J., Mitchell, S.E., Soares, A.M.V.M.. 2002c. Among and within population variability in tolerance to cadmium stress in natural populations of *Daphnia magna*:

implications for ecological risk assessment. Environmental Toxicology and Chemistry, 21(5): 1058-1065.

Barata, C., Baird, D.J., Soares, A.M.V.M.. 2002a. Determing genetic variability in the distribution of sensitivities of toxic stress among and within field populations of *Daphnia magna*. Environmental Science and Technology, 36(14): 3045-3049.

Barnes, R.D.. 1980. Invertebrate zoology. Moult Saunders College Publishing, Philadelphia.

Bennett, A.F.. 2003. Experimental evolution and the Krogh principle: Generating biological novelty for functional and genetic analyses. Physiological and Biochemical Zoology, 76(1): 1-11.

Bervoets, L., Blust, R.. 2003. Metal concentrations in water, sediment and gudgeon (*Gobio gobio*) from a pollution gradient: relationship with fish condition factor. Environmental Pollution, 126(1): 9-19.

Bickham, J.W., Smolen, M.J.. 1994. Somatic and heritable effects of environmental genotoxins and the emergence of evolutionary toxicology. Environmental health perspectives, 102: 25-28.

Bickham, J.W., SXandu, S., Hebert, P.D.N., Chicki, L., Athwal, R.. 2000. Effects of chemical contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. Mutation research- reviews in mutation research, 463 (1): 33-51.

Boersma M, De Meester L, Spaak P., 1999. Environmental stress and local adaptation in *Daphnia magna*. Limnology and oceanography, 44(2): 393-402.

Brausch, J.M., Smith, P.N.. 2009. Development of resistance to cyfluthrin and naphthalene among *Daphnia magna*. Ecotoxicology, 18(5): 600-609.

Bublyi, O.A., Loeschke, V.. 2002. Effect of low stressful temperature on genetic variation of five quantitative traits in Drosophila melanogaster. Heredity, 89: 70-75

Byers, D.L.. 2005. Evolution in heterogeneous environments and the potential of maintenance of genetic variation in traits of adaptive significance. Genetica, 123(1-2): 107–124.

Calow, P., Sibly, R.M., Forbes, V.. 1997. Risk assessment on the basis of simplified life-history scenarios. Environmental Toxicology and Chemistry, 16(9): 1983-1989.

Capaul, M., Ebert, D.. 2003. Parasite-mediated selection in experimental Daphnia magna populations. Evolution 57(2): 249-260.

Chapman, P.M.. 2008. Environmental risks of inorganic metals and metalloids: a continuing, evolving scientific odyssey. Human and Ecological Risk assessment 14: 5-40.

Chaumot, A, Gos, P., Garric, J., Geffard, O..2009. Additive vs non-additive genetic components in lethal cadmium tolerance of *Gammarus* (Crustacea): novel light on the assessment of the potential for adaptation to contamination. Aquatic Toxicology, 94:294-299

Colbourne, J.K., Singan, V.R., Gilbert, D.G. .2005. wFleaBase: The *Daphnia* genome database. BMC Bioinformatics, 6(45).

Conner, J.K.. 2003. Artificial selection: a powerful tool for ecologists. Ecology, 84 (7): 1650-1660.

Coors, A., Vanoverbeke, J., De Bie, T., De Meester, L.. 2009. Land-use, genetic diversity and toxicant tolerance in natural *Daphnia magna* populations. Aquatic Toxicology 95(1):71-79.

Cousyn, C., De Meester, L., Colbourne, J.K., Brendonck, L., Verschuren, D., Volckaert, F.. 2001. Rapid, local adaptation of zooplankton behaviour to changes in predation pressure in the absence of neutral genetic changes. Proceedings of the national Academy of sciences of the United States of America, 98 (11):6256-6260

Crommentuiyn, T., Stab, J.A., Estoppey, O., Vangestel, C.A.M.. 1995. Comparative ecotoxicity of cadmium, chlorpyrifos and triphenyltin hydroxide for 4 clones of the parthenogenetic collembolan *Folsomia candida* in an artificial soil. Functional ecology, 9(5): 734-742.

De Meester, L.. 1991. An analysis of the phototactic behaviour of *Daphnia magna* clones and their sexual descendants. Hydrobiologia, 225: 217-227.

De Meester, L., Weider, J., Tollrian, R.. 1995. Alternative antipredator defences and genetic polymorphism in a pelagic predator–prey system. Nature, 378: 483–485.

De Meester, L., Vanoverbeke, J., De Gelas, K., Ortells, R., Spaak, P.. 2006. Genetic structure of cyclic parthenogenetic zooplankton populations – a conceptual framework. Archives Hydrobiologia, 167 (1-4): 217-244.

Declerck, S., De Bie, T., Ercken, D., Hampel, H., Schrijvers, S., Van Wichelen, J., Gillard, V., Mandiki, R., Losson, B., Bauwens, D., Keijers, S., Vyverman, W., Goddeeris, B., De Meester, L., Brendonck, L., Martens, K.. 2006. Ecological characteristics of small farmland ponds: associations with land use practices at multiple spatial scales. Biological Conservation, 131 : 523-532.

Declerck, S., Vandekerkhove, J., Johansson, L., Muylaert, K., Conde-Porcuna, J.M., Van der Gucht, K., Perez-Martinez, C., Lauridsen, T., Schwenk, K., Zwart, G., Rommens, W., Lopez-Ramos, J., Jeppesen, E., Vyverman, W., Brendonck, L., De Meester, L.. 2005. Multi-group biodiversity in shallow lakes along gradients of phosphorus and water plant cover. Ecology, 86 : 1905-1915.

Deng, D.G., Zhang, S., Li, Y.Y., Meng, X.L., Yang, W., Li, Y., Li, X.X.. 2010. Effects of Microcystis aeruginosa on population dynamics and sexual reproduction in two Daphnia species. Journal of plankton research, 32(10): 1385-1392.

Dodson, S.I., Merritt, C.M., Shannahan, J.P., Schults, C.. 1999. Lower exposure concentrations of atrazine increase male production in *Daphnia pulicaria*. Environmental Toxicology and Chemistry, 18: 1568-1573.

Doroszuk, A., Wojewodzic, M.W., 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, 273: 2611-2618.

Ebert, D., Yamploski, L., Stearns, S.C.. 1993. Genetics of life history in *Daphnia magna*. I. Heritability at two food levels. Heredity, 70: 335-343.

ECB. 2007. European Union Risk Assessment Report on Cadmium metal / Cadmiumoxide. European Chemicals Bureau, Ispra, Italy

ECHA. 2008. Guidance on information requirements and chemical safety assessment Chapter R10: Characterisation of dose [concentration]-response for environment Related to REACH

Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 European Chemicals Agency, Helsinki, Finland.

Elendt, B.P., Bias, W.R.. 1990. Trace nutrient deficiency in *Daphnia magna* cultured in standard medium for toxicity testing. Effects of the optimization of culture conditions on life history parameters of *Daphnia magna*. Water research, 24(9): 1157-1167.

EU..2005. Environmental Quality Standards (EQS) Substance Data Sheet Priority Substance No 6 Cadmium and its Compounds Common Implementation Strategy for the Water Framework Directive Final Version, Brussels, July 31st, 2005.

EU..2008. Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy The Official Journal of the European Union, L348: 84-97 December 24th, 2008.

EU..2003. Technical Guidance Document on Risk Assessment Part IV in Support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances, Commission Regulation (EC) No 1488/94 on Risk Assessment for existing substances, and Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market. Institute for Health and Consumer Protection, European Chemicals Bureau, European Commission, Join Research Centre, Ispra, Italy, 328 p.

European Commission, 2006. Technical Guidance Document in Support of Commission Directive 93/67/EEC on Risk Assessment for New Notified Substances and Commission Regulation (EC) No 1488/94 on Risk Assessment for Exiting Substances, European Commission, Luxembourg (2006) ISBN 92-827-8011-2.

Falconer, D.S., Mackay, T.F.C.. 1996. Introduction to quantitative genetics. Pearson Education Limited, 4th edition, Londen. pp464.

Ferreira, A.L., Serra, P., Soares, A.M.V.M., Loureiro, S.. 2010. The influence of natural stressors on the toxicity of nickel to *Daphnia magna*. Environmental Science and Pollution Research, 17(6): 1217-1229.

Fisher, R.A..1930. The Genetical Theory of Natural Selection. Clarendon, Oxford.

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(7): 1544-1556.

Forbes, V.E., Depledge, M.H.1996. Environmental stress and the distribution of traits within populations. Ecotoxicology: Ecological Dimensions, eds DJ Baird, L Maltby, PW Greig-Smith, PET Douben, Chapman and Hall, Londen, pp71-76.

Forbes, V.E., Forbes, T.L.. 1994. Ecotoxicology in Theory and Practice. Chapman & Hall, London.

Forbes, V.E.. 1998. Genetics and Ecotoxicology. Taylor & Francis, Philadelphia, PA, USA.

Groenendijk, D., Kraak, M.H.S., Admiraal, W..1999a. Efficient shedding of accumulated metals during metamorphosis in metal-adapted populations of the midge *Chironomus riparius.* Environmental Toxicology and Chemistry, 18: 1225-1231.

Groenendijk, D., Van Opzeeland, B., Pires, L.M.D., Postma, J.F..1999b. Fluctuating life-history parameters indicating temporal variability in metal adaptation in riverine chironomids. Archives Environmental Contamination and Toxicology, 37: 175-181.

Haap, T., Kohler, H.R.. 2009. Cadmium tolerance in seven *Daphnia magna* clones is associated with reduced hsp 70 baseline levels and induction. Aquatic toxicology, 94 (2): 131-137.

Hebert, P.D.N.. 1987. Genotypic characteristics of the Cladocera. Hydrobiologia, 145: 183-193.

Heugens, E.H.W., Jager, T., Creyghton, R., Kraak, M.H.S., Hendriks, A.J., Van Straalen, N.M., Admiraal, W.. 2003. Temperature dependent effects of cadmium on *Daphnia magna*: Accumulation versus sensitivity. Environmental Science and Technology, 37: 2145-2151.

Heugens, E.H.W., Tokkie, L.T.B., Kraak, M.H.S., Hendriks, A.J., Van Straalen, N.M., Admiraal, W.. 2006. Population growth of *Daphnia magna* under multiple stress conditions: joint effects of temperature, food, and cadmium. Environmental toxicology and Chemistry, 25(5): 1399-1407.

Fisker, K.V., Sorensen, J.G., Damgaard, C., Pedersen, K.L., Holmstrup, M.. 2011. Genetic adaptation of earthworms to copper pollution: is adaptation associated with fitness costs in *Dendrobaena octaedra*? Ecotoxicology, 20: 563-573.

Hoffmann, A.A., Hercus, M.J.. 2000. Environmental stress as an evolutionary force. Bioscience, 50(3): 217-226.

Hoffmann, A.A., Merilä, J.. 1999. Heritable variation and evolution under favourable and unfavourable conditions. Trends in ecology and evolution, 14(3): 96-101.

Hoffmann, A.A., Parsons, P.A. 1991. Evolutionary genetics and Environmental Stress. Oxford University Press, New York.

Hooper, H.L., Connon, R., Callaghan, A., Fryer, G., Yarwood-Buchanan, S., Biggs, J., Maund, S.J., Hutchington, T.H., Sibly, R.M.. 2008. The ecological niche of *Daphnia magna* characterized using population growth rate. Ecology, 89(4): 1015-1022.

Houle, D.. 1992. Comparing evolvability and variability of quantitative traits. Genetics, 130(1): 195-204.

Jansen, M., Stoks, R., Coors, A., De Meester, L.. 2010. No evidence for a cost of selection by carbaryl exposure in terms of vulnerability to fish predation in *Daphnia magna.* Hydriobiologia, 643: 123-128.

Jansen, M., Coors, A., Stoks, R., De Meester, L.. 2011. Evolutionary ecotoxicology of pesticide resistance: a case study in *Daphnia*. Ecotoxicology, 20: 543-551.

Jensen, A., Forbes, V.E.. 2001. Interclonal variation in the acute and delayed toxicity of cadmium to the European prosobranch gastropod *Potamopyrgus antipodarum* (Gray). Archives of environmental contamination and toxicology, 40(2): 230-235.

Kawecki, T.J., Ebert, D.. 2004. Conceptual issues in local adaptation. Ecology letters, 7(12): 1225-1241.

Klerks, P.L., Moreau, C.J.. 2001. Heritability of resistance to individual contaminants and to contaminant mixtures in the sheepshead minnow (*Cyprinodon variegatus*). Environmental Toxicology and Chemistry, 20(8): 1746-1751.

Klerks, P.L., Weis, J.S.. 1987. Genetic adaptation to heavy-metals in aquatic organisms- a review. Environmental Pollution, 45 (3): 173-205.

Klerks, P.L.. 2002. Adaptation, ecological impacts, and risk assessment: insights from research at Foundry Cove, Bayou Trepagnier and Pass Fourchon. Human and Ecological Risk assessment 8(1): 971-982.

Klerks, P.L., Xie, L.T., Levinton, J.S.. 2011. Quantitative genetics approaches to study evolutionary processes in ecotoxicology: a perspective from research on the evolution of resistance. Ecotoxicology, 20(3): 513-523.

Klüttgen, B., Dülmer, U., Engels, M., Ratte, H.T.. 1994. Adam, an artificial freshwater for the culture of zooplankton. Water research 28: 743-746.

Levinton, J.S., Suatoni, E., Wallace, W.. 2003. Rapid loss of genetically based resistance to metals after the cleanup of a Superfund site. Proceedings of the national academy of sciences of the united states of America, 100(21): 12525-12525.

Lopes, I., Baird, D.J., Ribeiro, R.. 2004. Genetic determination of tolerance to lethal and sublethal copper concentrations in field populations of *Daphnia longispina*. Archives of Envrionmental Contamination and Toxicology, 46:43-51.

Lopes, I., Baird, D.J., Ribeiro, R.. 2006. Genetic adaptation to metal stress by natural populations of *Daphnia longispina*. Ecotoxicology and environmental safety, 63(2): 275-285.

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 (8): 1189-1197.

Lopes, I., Martins, N., Baird, D.J., Ribeiro, R.. 2009. Genetic erosion and population resilience in *Daphnia longispina* under simulated predation and metal pressures. Environmental Toxicology and Chemistry, 28(9): 1912-1919.

Lotka A.. 1913. A natural population norm. Journal of the Washington Academy of Sciences 2: 241-248.

Lynch, M., Latta, L., Hicks, J., Giorgianni, M.. 1998. Mutation, selection, and the maintenance of life-history variation in a natural population. Evolution, 52(3): 727-733.

Lynch, M., Walsh, B.. 1998. Genetics and Analysis of Quantitative Traits. Sinauer Associates Inc., Sunderland, MA.

Mareus, N.H.. 1990. Calanoid copepod, cladoceran, and rotifer eggs in sea bottom sediments of northern Californian coastal waters: identification, occurrence and hatching. Marine Biology 105: 413-418.

Medina, M.H., Correa, J.A., Barata, C.. 2007. Micro-evolution due to pollution: Possible consequences for ecosystem responses to toxic stress. Chemosphere, 67(11): 2105-2114.

Messiaen, M., De Schamphelaere, K.A.C., Muyssen, B.T.A., Janssen, C.R..2010 Micro-evolutionary response of *Daphnia magna* population exposed to temperature and cadmium stress. Ecotoxicology Environmental Safety, 73(6): 1114-1122.

Meyer, J.N., Di Giuliuo, R.T.. 2003. Heritable variation and fitness costs in killifish (*Fundulus beteroclitus*) inhabiting a polluted estuary. Ecological applications, 13(2): 490-503.

Michels, E., Cottenie, K., Neys, L., De Meester, L.. 2001. Zooplankton on the move: first results on the quantification of dispersal of zooplankton in a set of interconnected ponds. Hydrobiologia, 442 : 117-126.

Millwards, R.N., Klerks, P.L.. 2002. Contaminant-adaptation and community tolerance in ecological risk assessment: introduction. Human and ecological risk assessment, 8 (5): 921-932.

Miyo, T., Akai, S., Oguma, Y.. 2000. Seasonal fluctuations in susceptibility to insecticides within natural populations of *Drosophila melanogaster*: empirical observations of fitness costs of insecticide resistance. Genes and Genetic systems, 75(2): 97-104.

Morgan, A.J., Kille, P., Stürzenbaum, S.R.. 2007. Microevolution and ecotoxicology of metals in Invertebrates. Environmental Science and Technology, 41(4): 1085-1096.

Muylaert, K., Declerck, S., Geenens, V., Van Wichelen, J., Degans, H., Vandekerkhove, J., Van der Gucht, K., Vloemans, N., Rommens, W., Rejas, D., Urrutia, R., Sabbe, K., Gillis, M., Decleers, K., De Meester, L., Vyverman, W.. 2003. Zooplankton, phytoplankton and the microbial food web in two turbid and two clearwater shallow lakes in Belgium. Aquatic Ecology, 37: 137-150.

Nowak, C., Czeikowitz, A., Vogt, C., Oetken, M., Streit, B., Schwenk, C.. 2008. Variation in sensitivity to cadmium among genetically characterized laboratory strains of the midge *Chironomus riparius*. Chemosphere, 71(10): 1950-1956.

OECD (2006) Draft Guidance Document for on the Statistical Analysis of Ecotoxicity Data

Onbe, T..1978. Sugar flotation method for sorting the resting eggs of marine cladocerans and copepods from sea-bottom sediment. Bulletin Japan Society of Science and Fisheries, 44: 1411.

Organization for Economic Cooperation and Development. *Daphnia magna* reproduction test. Guidelines for the testing of chemicals No. 211, 1998, Paris, France.

Peterson, J.K., Kashian, D.R., Dodson, S.I.. 2001. Methoprene and 20)OHecdysone affect male production in *Daphnia pulex*. Environmental Toxicology and Chemistry, 20: 582-588.

Picado, A., Chankov, S., Fernande, A., Fernand, S., Leverett, D., Johnso, I., Hernan, R., Pire, A.M., Matos, J.. 2007. Genetic variability in *Daphnia magna* and ecotoxicological evaluation. Ecotoxicology and Environmental Safety, 67:406-410.E

Postma J.F., Kyed, M., Admiraal, W..1995b. Site specific differentiation in metal tolerance in the midge *Chironomus riparius* (Diptera, Chironomidae). Hydrobiologia, 315:159-165.

Postma, J.F., Kleunen, A., Admiraal, W..1995c. Alterations in life-history traits of *Chironomus riparius* (Diptera) obtained from metal contaminated rivers. Archives of Environmental Contamination and Toxicology, 29: 469-475.

Postma, J.F., Mol, S., Larsen, H., Admiraal, W.. 1995a. Life-cycle changes and zinc shortage in cadmium—tolerant midges, *Chironomus riparius* (Diptera), reared in the absence of cadmium. Environmental Toxicology and Chemistry, 14(1): 117-122.

Postma, J.F., Van Nugteren, P., Buckert-de Jong, M.B.. 1996. Increased cadmium excretion in metal-adapted populations of the midge *Chironomus riparius* (Diptera). Environmental Toxicology and Chemistry, 15: 32-339.

Reznick, D., Nunney, L., Tessier, A.. 2000. Big houses, big cars, superfleas and the cost of reproduction. Trends in Ecology and Evolution, 15(10): 421-425.

Rodriguez, E.M., Medesani, D.A., Fingerman, M.. 2007. Endocrine disruption in crustaceans due to pollutants: a review. Comparative biochemistry and physiology a molecular and integrative physiology, 146: 661-671.

Roff, D.A., Fairbairn, D.J.. 2007. The evolution of trade-offs: where are we? Journal of Evolutionary Biology, 20(2): 433-447.

Salice, C.J., Anderson, T.A., Roesijadi, G.. 2010. Adaptive responses and latent costs of multigeneration cadmium exposure in parasite resistant and susceptible strains of a freshwater snail. Ecotoxicology, 19(8): 1466-1475.

Schwaegerle, K.E., Mclintyre, H., Swingley, C.. 2000. Quantitative genetics and the persistence of environmental effects in clonally propagated organisms. Evolution, 54(2): 452-461.

Searle, S.R., Casella, G., McCulloh, C.E.. 1992. Variance components. John Wiley and Sons, NY

Sgrò, 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(1):13-18.

Sgrò, C.M., Hoffmann, A.A.. 2004. Genetic correlations, trade-offs and environmental variation. Heredity, 93(3): 241-248.

Shirley, M.D.F., Sibly, R.M.. 1999. Genetic basis of a between-environment trade-off involving resistance to Cd in *Drosophila melanogaster*. Evolution, 53(3): 826-836.

Sun, L.; Perdue, E.M.; McCarthy, J.F.. 1995. Using reverse osmosis to obtain organic matter from surface and ground waters. Water Research, 29(6): 1471-1477.

Swindell, W.R., Bouzat, J.L.. 2006. Associations between environmental stress, selection history, and quantitative genetic variation in *Drosophila melanogaster.* Genetica 127(1-3): 311-320.

Templeton, A.R.. 2006. Population genetics and micro-evolutionary theory. John Wiley & Sons, Hoboken, New Jersey

Tipping, E.. 1994. WHAM-a chemical equilibrium model and computer code for waters, sediments, and soils incorporating a discrete site/electrostatic model of ion-binding by humic substances. Computers and Geosciences, 20(6): 937-1023.

US-EPA, 2001. Update of ambient water quality criteria for cadmium. EPA-882-R-01-001.

Vanoverbeke, J., De Gelas, K., De Meester, L.. 2007. Habitat size and the genetic structure of a cyclical parthenogen, *Daphnia magna*. Heredity, 98: 419-426.

Van Doorslaer, W., Stoks, R., Jeppesen, E., De Meester, L..2007. Adaptive micro-evolutionary responses to simulated global warming in *Simocephalus vetulus*: a mesocosm study. Global Change Biology, 13(4): 878-886.

Van Doorslaer, W., Stoks, R., Swillen, I., Freuchtmayr, H., Atkinson, D., Moss, B., De Meester, L.. 2010.Experimental thermal microevolution in community-embedded *Daphnia* populations. Climate research, 43(1-2): 81-89.

Van Doorslaer, W., Vanoverbeke, J., Duvivier, C., Rousseaux, S., Jansen, M., Jansen, B., Feuchtmayr, H., Atkinson, D., Moss, B., Stoks, R., De Meester, L.. 2009. Local adaptation to

higher temperatures reduces immigration success of genotypes from a warmer region in the water flea *Daphnia magna*. Global change biology, 15(12): 3046-3055.

Van Ewijk, P.H. and Hoekstra, J.A.. 1993. Calculation of the EC_{50} and its confidence interval when subtoxic stimulus is present. Ecotoxicology and Environmental Safety, 25: 25–32.

Van Straalen NM.. 2003. Ecotoxicology becomes stress ecology. Environmental Science and Technology, 37(17): 324A-330A.

Van Straalen, N.M., Timmermans, M.J.. 2002. Genetic variation in toxicant-stressed populations: an evaluation of the "Genetic Erosion" hypothesis. Human and Ecological Risk Assessment, 8(5): 983-1002.

Vandegehuchte, M.B., Kyndt, T., Vanholme, B., Haegeman, A., Gheysen, G., Janssen, C.R.. 2009. The occurrence of DNA methylation in *Daphnia magna* and influence of multigenerational Cd exposure. Environmental International, 35(4): 700-706.

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(3): 188-195.

Vandekerkhove, J., Declerck, S., Vanhove, M., Brendonck, L., Jeppesen, E., Conde Porcuna, J.M., De Meester, L.. 2004. Use of ephippial morphology to assess richness of anomopods: potentials and pitfalls. Journal of limnology, 63(1): 75-84

Vanoverbeke, J., De Meester, L.. 2010. Clonal erosion and genetic drift in cyclical parthenogens – the interplay between neutral and selective processes. Journal of evolutionary biology, 23(5): 997-1012.

Via, S.. 1984. The quantitative genetics of polyphagy in an insect herbivore. I. Genotype-environment interaction in larval performance on different host plant species. Evolution, 38(4): 881-895.

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

Vidal, D.E., Horne, A.J.. 2003b. Mercury toxicity in the aquatic oligochaete *Sparganophilus persaei*: I. Variation in resistance among populations. Archives of environmental Contamination and toxicology, 45: 184-189.

Von der Ohe, P.C., Liess, M.. 2004 Relative sensitivity distribution of aquatic invertebrates of organic and metal compounds. Environmental Toxicology and Chemistry, 23(1):150-156.

Ward T.J., Robinson, W.E.. 2005. Evolution of cadmium resistance in *Daphnia magna*. Environmental Toxicology and Chemistry, 24(9): 2341-2349.

Warming, T.P., Mulderij, G., Christoffersen, K.S.. 2009. Clonal variation in physiological responses of *Daphnia magna* to the strobilurin azoxystrobin. Environmental Toxicology and Chemistry, 28(2): 374-380.

Wogram, J., Liess, M.. 2001. Ranking order of macro-invertebrate sensitivity to toxic compounds by comparison with that of *Daphnia magna.* Bulletin of Environmental Contamination and Toxicology, 67(3): 360-367.

Xie, L.T., Klerks, P.L.. 2003. Responses to selection for cadmium resistance in the least killifish, *Heterandria formosa*. Environmental Toxicology and Chemistry, 22(2): 313-320.

Zbinden, M., Haag, C.R., Ebert, D.. 2008. Experimental evolution of field populations of *Daphnia magna* in response to parasite treatment. Journal of evolutionary biology 21(4): 1068-1078.

Summary

The ultimate goal of environmental risk assessment is to prevent chemical substances causing irreversible damages to ecosystems. However, in conventional ecotoxicology, tolerance to stress is tested under standardized laboratory conditions and is also concerned with the short-term effects of stressors. By using, for example monoclonal *D. magna* populations or inbred populations, genetic variability is minimized, which increases the precision of the estimation of mean population responses and also decreases the variability among toxicity tests. Field populations, however, may be exposed to long-term chemical stress and do exhibit genetic variation. Both factors have mostly been ignored in routine ecotoxicology and environmental risk assessment. Field populations which experience an initially reduced fitness due to chemical exposure may exhibit phenotypic variation of fitness among individuals upon which natural selection may act. If this variation is also heritable - i.e. if it contains a significant genetic component - micro-evolutionary changes in the genetic make-up of the population may result in an increase of the mean fitness of the population. Thus, natural selection may result in genetic adaptation of a population to pollution. This genetic adaptation can be seen as an positive event. However, other micro-evolutionary aspects should also be taken into consideration, i.e cost of tolerance and fitness trade-offs. The effect of Cd on these micro-evolutionary aspects, i.e.(1) micro-evolutionary potential, (2) cost of tolerance and (3) fitness trade-offs can be revealed by using quantitative genetic studies and micro-evolutionary experiments.

Daphnia magna populations are the ideal test-organisms for those study types of micro-evolutionary responses. They are widely used in risk assessment and they can produce asexually by ameiotic parthenogenesis, thus the genetic and environmental components of variance can be separated in experimental design. Additionally *Daphnia* (i) are amongst the

freshwater species which are most sensitive to chemicals, (ii) have demonstrated to exhibit genetic variability of Cd tolerance within populations, (iii) have been demonstrated to rapidly adapt to stress, and (iv) provide ideal model species to study both genetic variation and micro-evolutionary responses in populations.

In chapter 2, we determined the genetic coefficient of variation (CV_G) and broad sense heritability (H^2) as measures of micro-evolutionary potential of total reproduction (R_0) and population growth rate (r_m) by means of 21-day life-table experiments with 11 genetically distinct clones from natural *Daphnia magna* population exposed to a control and Cd concentrations between 0.89 and 18.9 µg Cd/L. We also determined a cost of tolerance (i.e. negative genetic correlations between environments) within this Cd range. Based on significantly higher genetic variation of fitness in a Cd treatment vs. the control, a higher micro-evolutionary potential was observed at at 18.9 µg Cd/L (based on $CV_G(R_0)$, $CV_G(r_m)$, and $H^2(r_m)$). No cost of Cd tolerance in higher Cd environments was found.

After detecting an increased micro-evolutionary response under Cd stress, we examined micro-evolutionary aspects of a natural *Daphnia magna* population exposed to Cd and to how these are influenced by temperature. In chapter 3, life-table experiments were conducted with 14 *D. magna* clones collected from an unpolluted lake following a 2×2 design with Cd concentration and temperature as the factors (control vs. 5 µg/L cadmium, 20°C vs. 24°C). Our results demonstrate (1) that chemicals can have effects on key population genetic characteristics such as genetic variation and between-trait correlations and (2) that these effects may differ depending on temperature. They findings also suggests that further research is needed to understand the importance of combined chemical - global warming stress for micro-evolutionary responses of organisms.

The above studies determined the micro-evolutionary potential of a population towards stress. As this genetic variability embraces also non-additive genetic components, this genetic variability could overestimate this potential for adaptation. In chapter 4, we examined the additive and non-additive components of a natural *Daphnia magna* population exposed to Cd stress and how these are influenced by temperature. Life-table experiments were conducted with 20 parent and 39 offspring clones following a 2×2 design with Cd concentration and temperature as the factors (control vs. 5 µg/L cadmium, 20°C vs. 24°C). Total reproduction and population growth rate were monitored during 21 days. Variance components were determined using an Animal Model. Our results indicate that temperature and Cd can have significant effects on additive and non-additive components of a *Daphnia magna* population. The finding of a significant additive genetic variance in the 24°C + Cd treatment, indicates that genetically determined differences for Cd stress in combination with temperature may be heritable to the next generations.

In the above three chapters, we focused mainly on one *Daphnia magna* population, although variability among *Daphnia magna* populations could influence the outcome of our results. In chapter 5, we examined the between and within genetic variability of 11 natural *Daphnia magna* population exposed to a sublethal Cd stress. Life-table experiments with a control and Cd treatment (5 µg Cd/L) were conducted with 12 *D. magna* clones originating from 11 *Daphnia magna* populations collected from 11 (Cd) unpolluted lakes. Several fitness traits were monitored during 21 days. Our results indicate that there is between and within genetic variability in *Daphnia magna* populations, indicating that populations originating from other habitats may have a different micro-evolutionary potential under Cd stress.

In the previous chapter, we estimated micro-evolutionary potential and micro-evolutionary constrains by using quantitative genetics. In chapter 6, a 203-day during micro-evolutionary experiment was conducted to test the micro-evolutionary response in a *Daphnia magna* population exposed to a Cd range between 0 and 20.77 µg Cd/L under semi-field conditions. There was a response observed at 17.83 µg Cd/L, as the long-term 20.77 µg Cd/L exposed population had a higher fitness under Cd stress than (1) the population exposed under control conditions (0 µg Cd/L) and (2) the start population (population kept under laboratory conditions). However, genetic drift could not be excluded. Besides an increased tolerance, no associated costs were observed.

In chapter 7, we compared our found results with the conventionally derived PNEC and EQS for Cd. Additionally, we determined conventially derived NOECs from 7 European monoclonal *Daphnia magna* populations. In conclusion, all the evidence so far indicates that an increased micro-evolutionary response to Cd exposure only occur above the conventionally derived PNEC and HC_5 of Cd. However, given the large differences between populations of the same species and between different species, it is recommended to broaden the knowledge to more *D. magna* populations and to additional species as well. In addition, the fact that it is theoretically possible that PNEC and EQS derived from a single *D. magna* NOEC may be higher than the concentration at which micro-evolutionary effects may be possible, calls to extend this kind of research to multiple chemicals.

Measure of micro-evolutionary change	Test organism	Chapter	Stressor	Research-questions	Result
Quantitative genetics	1 *Daphnia magna* population	2	Cd concentration range: 0-18.9 µg Cd/L	Cd effect on population mean? Cd effect on micro-evolutionary potential? Is there a cost of tolerance?	NOEC of 0.9 µg Cd/L Increased micro-evolutionary potential at increasing Cd stress. Significant increased $CV_G(R_0)$ at 18.9 µg Cd/Lcompared to the control No cost of tolerance
		3	Control and 4.1 µg Cd/L under two temperature treatments (20°C and 24°C)	Is there a temperature effect on micro-evolutionary aspects? - Increased temperature result in a larger Cd effect? - Increased stress results in an increased micro-evolutionary potential under Cd stress? - Is there a cost of tolerance? - Among fitness-traits genetic correlations?	- Temperature results in a larger Cd effect compared to the control - Temperature results in an increased micro-evolutionary potential at 4.1 µg Cd/L compared to the control - No cost of tolerance - Cd effect on trade-offs
		4	Control and 3.8 µg Cd/L under two temperature treatments	Is there a temperature and Cd effect on: - Additive components - Dominance components - h^2	Significant additive genetic variance and h^2 found at 24°C + Cd
	11 *Daphnia magna* populations	5	Control and 4.3 µg Cd/L	Cd effect on micro-evolutionary potential of 11 *Daphnia magna* populations?	45% of the populations had a significant increased micro-evolutionary potential at 4.3 µg Cd/L
Micro-evolutionary experiment	1 *Daphnia magna* population	6	Long-term Cd exposure between 0-20.77 µg Cd/L	At which Cd concentration is there a rapd micro-evolutionary response? Is there a cost of tolerance?	Increased population fitness under 17.3 µg Cd/L found in long-term 20.77 µg Cd/L exposed population No cost of tolerance

Chapter 7

1. Are there micro-evolutionary effects at conventionally derived PNEC and EQS? NO
2. Are there micro-evolutionary effects at conventionally derived NOEC's (use of monoclonal laboratory *Daphnia magna* populations)? This may be possible

Samenvatting

Het doel van omgeving risk assessment is het voorkomen van een onomkeerbare schade toegebracht aan het milieu door chemische stoffen. In routinematige ecotoxicologie wordt tolerantie aan stress getest onder gestandardizeerde labo-omstandigheden. Dit wordt uitgevoerd door o.a. gebruik te maken van monoklonale *Daphnia magna* populaties, waardoor genetische variabiliteit wordt geminimalizeerd. Daardoor wordt een betere en preciezere schatting van de gemiddelde populatie responsen bekomen en wordt de variabiliteit tussen test resultaten verminderd. Veldpopulaties hebben daarentegen genetische variabiliteit en kunnen ook blootgesteld worden gedurende lange tijd aan chemische stress. Veldpopulaties die een gereduceerde fitness hebben door chemische blootstelling kunnen een phenotypische variatie hebben tussen individuen waarop selectie kan inwerken. Indien deze variatie overerfbaar is, kunnen micro-evolutionaire veranderingen in de genetische opmaak van de populatie leiden tot een stijging van de fitness van die populatie. Dus, natuurlijke selectie leidt tot genetische adaptatie van een populatie blootgesteld aan een chemische stof. Deze adaptatie kan gezien worden als een “positief” effect. Maar andere micro-evolutionaire aspecten zoals kost aan adaptatie an fitness trade-offs moeten ook in rekening gebracht worden. Het effect van Cd op deze micro-evolutionaire aspecten: (1) potentiaal voor micro-evolutie, (2) kost aan adaptatie en (3) fitness trade-offs kan onderzocht worden aan de hand van kwantitatieve genetica en micro-evolutionaire experimenten.

Daphnia magna populaties zijn de ideale test organismen voor deze studies van micro-evolutionaire responsen. Deze watervlo wordt gebruikt in risk assessment en door hun asexuele voortplanting kunnen de genetische en omgevings componenten van variatie onderscheiden worden. Daarenboven zijn Daphnia’s (1) gevoelig voor chemische stressoren,

(2) hebben ze genetische variabiliteit voor Cd tolerantie in populaties, (3) kunnen ze vlug adapteren aan stress en (4) zijn deze het ideale test organisme om genetische variatie en micro-evolutionaire responsen te onderzoeken in populaties.

In hoofdstuk 2 bepaalden we de genetische coefficient van variatie (CV_G) en overerfbaarheid s.l. (H^2) als parameters voor micro-evolutionair potentiaal van totale reproductie en populatie groeisnelheid. Daarbij werden 21 dagen experimenten uitgevoerd met 11 genetisch verschillende klonen van een natuurlijke *Daphnia magna* populatie blootgesteld aan een controle en een Cd concentratie range tussen 0.9 en 18.9 µg Cd/L. Er werd ook een kost aan adaptatie bepaald. Gebaseerd op de hogere genetische variatie in fitness in een Cd behandeling vergeleken met de controle, werd een hoger potentiaal voor micro-evolutie gevonden bij 18.9 µg Cd/L (gebaseerd op $CV_G(R_0)$, $CV_G(r_m)$ en $H^2(r_m)$). Geen kost aan adaptatie werd teruggevonden.

In een volgend hoofdstuk werden de verschillende micro-evolutionaire aspecten van deze *D. magna* populatie nagegaan, blootgesteld aan Cd en onder invloed van temperatuursverhoging. In hoofdstuk 3, werden experimenten uitgevoerd met 14 *D. magna* klonen volgens een 2x2 design met Cd concentratie en temperatuur als factoren (controle vs 5 µg Cd/L; 20°C vs 24°C). Onze resultaten toonden aan dat (1) chemische stoffen effecten hebben op genetische populatie kenmerken zoals genetische variatie en tussen-trait correlaties en (2) deze effecten afhankelijk zijn van temperatuur. Deze bevindingen suggereren dat verder onderzoek noodzakelijk is om het effect van de combinatie 'toxicant-klimaatsverandering' op micro-evolutionaire responsen bij populaties na te gaan.

De voorafgaande studies bepaalden de micro-evolutionaire potentiaal van een populatie. Deze genetische variabiliteit heeft echter ook niet-additieve componenten,

waardoor deze genetische variabiliteit een overschatting kan zijn van de potentiaal tot adaptatie. In hoofdstuk 4 onderzochten we de additieve en niet-additieve componenten van een natuurlijke *D. magna* populatie blootgesteld aan Cd stress en hoe deze beïnvloed werden door temperatuur. Experimenten werden uitgevoerd met 20 ouder klonen en 39 klonen van nakomelingen volgens een 2x2 design met Cd en temperatuur als factoren. Totale reproductie en populatie groeisnelheid werden opgevolgd gedurende 21 dagen. Variantie componenten werden bepaald door gebruik te maken van het 'Animal Model'. Onze resultaten toonden aan dat temperatuur en Cd significante effecten hebben op de additieve en niet-additieve componenten van een *D. magna* populatie. Het vinden van een significante additieve genetische variatie in de 24°C + Cd behandeling duidde aan dat genetisch gedetermineerde verschillen voor Cd stress in combinatie met temperatuur overerfbaar kunnen zijn naar de volgende generaties.

Hoofdstukken 2 tot en met 4 maakten gebruik van één *Daphnia magna* populatie, alhoewel genetische variabiliteit tussen populaties een invloed kan hebben op de interpretatie van onze resultaten. In hoofdstuk 5 werd de genetische variabiliteit tussen en in 11 populaties nagegaan. Experimenten werden uitgevoerd met een controle en Cd behandeling (5 µg Cd/L) met 11 populaties elk bestaande uit 12 klonen. Verschillende fitness kenmerken werden opgevolgd gedurende 21 dagen. Onze resultaten toonden aan dat er genetische variabiliteit is tussen populaties maar ook binnenin de populaties. Dit geeft aan dat populaties afkomstig van verschillende habitats een ander micro-evolutionair potentiaal onder Cd stress kunnen hebben.

De voorafgaande hoofdstukken maakten gebruik van kwantitatieve genetica. In hoofdstuk 6, werd een 203 dagen durend micro-evolutionair experiment uitgevoerd. Daarbij

werd de micro-evolutionaire respons nagegaan van een *D. magna* populatie blootgesteld aan een Cd concentratie range tussen 0 en 20.8 µg Cd/L. Nadien werd een respons teruggevonden bij 17.8 µg Cd/L, waarbij de populatie blootgesteld gedurende lange tijd aan de 20.8 µg Cd/L een hogere fitness had onder Cd stress dan de (1) start populatie en (2) de populatie blootgesteld onder controle omstandigheden. Alhoewel genetische drift niet volledig kon worden uitgesloten. Naast een verhoogde tolerantie werd geen kost aan adaptatie teruggevonden.

In het laatste hoofdstuk, hoofdstuk 7, vergeleken we onze resultaten met de conventioneel bepaalde PNEC en EQS voor Cd. Bijkomend werden de NOEC's van 7 Europese monoklonale *D. magna* populaties bepaald. Samengevat blijkt dat een verhoogde micro-evolutionaire respons teruggevonden werd boven de PNEC en HC_5 van Cd. Maar, door de grote verschillen tussen populaties maar ook tussen verschillende soorten, wordt het aanbevolen om de kennis uit te breiden naar meerdere soorten en populaties. Daarenboven, door het feit dat het theoretisch mogelijk is dat een PNEC en EQS bekomen door één *D. magna* NOEC hoger is dan de concentratie waarbij micro-evolutionaire effecten kunnen plaatsvinden is dit soort onderzoek bij andere chemische stoffen noodzakelijk.

Curriculum vitae

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Publications

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Vandendriessche S., Messiaen M., O'Flynn S., Vincx M., Degraer S.. 2006. Hiding and feeding in floating seaweed: Floating seaweed clumps as possible refuges or feeding grounds for fishes. Estuarine coastal and shelf science 71 (3-4): 691-703.

Messiaen M., De Schamphelaere K.A.C., Muyssen B.T.A., Janssen C.R.. 2010. The micro-evolutionary potential of a *Daphnia magna* population exposed to temperature and cadmium stress. Ecotoxicology and Environmental Safety 73(6): 1114-1122.

Muyssen B.T.A., Messiaen M., Janssen C.R.. 2010. Combined cadmium and temperature acclimation in *Daphnia magna*: physiological and subcellular effects. Ecotoxicology and Environmental Safety 73(5): 735-742.

Platform presentations (presenting author)

Messiaen, M., De Schamphelaere, K.A.C., Muyssen, B.T.A., Janssen, C.R.. 2008. Chronic ecotoxicity of cadmium to *Daphnia longispina* exposed in very soft surface water. SETAC Europe 18th annual meeting, Warsaw, Poland

Messiaen, M., Rousseaux, S., De Meester, L., Balcaen, L., Vanhaecke, F., Janssen, C.R., De Schamphelaere, K.A.C.. 2010. Which habitat characteristics explain within and between population differences in Cd tolerance of *Daphnia magna*? SETAC Europe 20th annual meeting, Sevilla, Spain

Poster Presentations (presenting author)

Messiaen, M., De Schamphelaere, K.A.C., Muyssen, B.T.A., Janssen, C.R.. 2008. Heritability of different fitness traits of *Daphnia magna* population under multiple stress. SETAC Europe 18th annual meeting, Warsaw, Poland

Messiaen, M., De Schamphelaere, K.A.C., Muyssen, B.T.A., Janssen, C.R.. 2009. Micro-evolutionary response of *Daphnia magna* population exposed to cadmium and temperature stress. SETAC Europe 19th annual meeting, Goteborg, Sweden

Messiaen, M., De Schamphelaere, K.A.C., Muyssen, B.T.A., Janssen, C.R.. 2009. Narrow sense heritability of fitness traits in a *Daphnia magna* population under multiple stress. Poster and spotlight presentation, SETAC Europe 19th annual meeting, Goteborg, Sweden

Messiaen, M., De Schamphelaere, K.A.C., Muyssen, B.T.A., Janssen, C.R.. 2009. A novel type of concentration response curve for Cd based on genetic variability among *Daphnia magna* clones. SETAC Europe 19th annual meeting, Goteborg, Sweden

Messiaen, M., De Schamphelaere, K.A.C., Muyssen, B.T.A., Janssen, C.R.. 2009. A novel type of concentration response curve for Cd based on genetic variability among *Daphnia magna* clones. ESEB, Turin, Italy.

Messiaen, M., Rousseaux, S., De Meester, L., Balcaen, L., Vanhaecke, F., Janssen, C.R., De Schamphelaere, K.A.C.. 2010: Do natural and human stressors explain the observed Cd tolerance among and within *Daphnia magna* population? Daphnia Genomics Consortium, Leuven, Belgium.

Muyssen, B.T.A., Messiaen, M., Janssen, C.R. 2010. Multi-generation temperature and cadmium acclimation in *Daphnia magna*: consequences for the stress response during combined exposures. SETAC Europe 20th annual meeting, Sevilla, Spain

Membership of professional organizations

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Supplementary Material

Table S2.1: Physico-chemical characteristics of test media during the Cd exposure experiment. Values represent mean ± standard deviation. NM is new medium. OM is old medium.

Nominal Cd concentration (µg Cd/L)		DOC (mg C/L)	pH	Cd concentration (µg Cd/L)	Mean Cd concentration (µg Cd/L)
0	NM	4.62	7.67	<0.1	<0.1
	OM	5.45 ± 0.64	7.58 ± 0.22	<0.1	
1	NM	4.69	7.63	1.06 ± 0.05	0.89 ± 0.22
	OM	5.74 ± 0.34	7.60 ± 0.24	0.73 ± 0.25	
2.2	NM	4.84	7.65	1.96 ± 0.18	1.92 ± 0.06
	OM	5.83 ± 0.52	7.70 ± 0.31	1.88 ± 0.38	
4.6	NM	4.86	7.62	4.56 ± 0.16	3.96 ± 0.88
	OM	5.99 ±0.30	7.73 ± 0.25	3.34 ± 1.37	
10	NM	4.69	7.65	9.79 ± 0.01	8.34 ± 1.99
	OM	5.97 ± 0.67	7.76 ± 0.27	6.94 ± 2.38	
22	NM	4.76	7.66	20.60 ± 0.42	18.87 ± 2.45
	OM	5.89 ± 1.32	7.86 ± 0.27	17.13 ± 1.92	

Table S2.2: Values of total reproduction (R_0) at control (0 µg Cd/L).

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	61	98	72	56	76	31	105	89	127	75	43
2	124	86	73	120	0	43	106	94	109	66	0
3	100	94	71	29	59	29	73	97	107	59	86
4	92	100	71	0	65	38	53	98	116	76	75
5	105	89	75		69	74	120	85	117	85	59
6	120		83		79		21	89	88	96	51

Table S2.3: Values of total reproduction (R_0) at 1 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	47	95	59	65	77	75	81	48	133	123	54
2	86	88	78	0	45	66	37	61	55	61	58
3	98	83	71	98	48	76	82	61	48	63	65
4	89	92	77	0	83	75	75	65	59	31	57
5	109	97	83		63	89	108	61	61	0	42
6	124	95	84		65		113	60	58	59	0

Table S2.4: Values of total reproduction (R_0) at 2.2 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	51	0	70	52	85	60	63	94	113	66	61
2	78	87	81	0	81	66	91	102	108	0	0
3	108	10	56	54	67	60	87	88	93	56	18
4	49	108	74	0	91	36	84	104	60	99	84
5	76	65	67		57	67	71	60	71	0	74
6	87	98	17		49		78	98	94	76	0

Table S2.5: Values of total reproduction (R_0) at 4.6 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	96	0	62	74	23	53	14	104	75	13	56
2	47	91	28	24	0	13	67	102	81	81	60
3	26	93	34	78	45	54	0	110	70	80	53
4	90	70	16	0	14	17	50	128	95	77	65
5	80	76	93		60	55	33	115	53	94	0
6	109	89	60		24		61	106	86	87	0

Table S2.6: Values of total reproduction (R_0) at 10 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	69	82	15	72	26	19	54	92	59	81	47
2	57	82	17	53	0	40	34	111	59	59	0
3	42	56	21	13	19	20	32	108	15	74	47
4	24	81	20	0	1	14	26	0	63	0	44
5	14	81	29		0	34	16	113	45	43	34
6	18	88	51		9		19	80	21	41	14

Table S2.7: Values of total reproduction (R_0) at 22 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0	0	9	8	0	0	3	8	0	8	9
2	0	9	8	2	0	2	0	13	0	13	0
3	0	22	2	0	4	6	0	22	7	7	11
4	0	14	9	0	0	8	1	19	13	15	0
5	10	19	4		2	11	0	19	0	17	0
6	5	10	22		5		0	10	0	11	0

Table S2.8: Values of population growth rate (r_m) at control (0 µg Cd/L).

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.35	0.41	0.32	0.35	0.40	0.32	0.40	0.35	0.36	0.38	0.33
2	0.38	0.37	0.34	0.36	0.00	0.34	0.40	0.35	0.39	0.36	0.00
3	0.40	0.39	0.33	0.34	0.34	0.30	0.40	0.39	0.36	0.34	0.38
4	0.41	0.38	0.35	0.00	0.35	0.32	0.34	0.36	0.36	0.38	0.37
5	0.40	0.38	0.35		0.35	0.32	0.37	0.38	0.38	0.37	0.28
6	0.39		0.35		0.40		0.32	0.35	0.34	0.42	0.31

Table S2.9: Values of population growth rate (r_m) at 1 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.29	0.42	0.33	0.37	0.37	0.32	0.40	0.32	0.36	0.38	0.36
2	0.35	0.41	0.36	0.00	0.34	0.35	0.37	0.34	0.36	0.35	0.38
3	0.34	0.36	0.32	0.35	0.34	0.35	0.40	0.35	0.34	0.34	0.37
4	0.38	0.40	0.36	0.00	0.37	0.32	0.41	0.39	0.32	0.37	0.36
5	0.38	0.40	0.37		0.34	0.34	0.35	0.38	0.31	0.00	0.30
6	0.37	0.40	0.36		0.31		0.35	0.34	0.35	0.37	0.00

Table S2.10: Values of population growth rate (r_m) at 2.2 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.35	0.00	0.34	0.31	0.40	0.33	0.36	0.33	0.35	0.35	0.36
2	0.32	0.39	0.36	0.00	0.36	0.35	0.39	0.34	0.38	0.00	0.00
3	0.39	0.29	0.32	0.28	0.34	0.35	0.40	0.37	0.38	0.28	0.32
4	0.33	0.39	0.36	0.00	0.35	0.29	0.42	0.40	0.31	0.36	0.36
5	0.39	0.37	0.35		0.36	0.30	0.35	0.39	0.32	0.00	0.41
6	0.36	0.36	0.32		0.33		0.37	0.34	0.38	0.37	0.00

Table S2.11: Values of population growth rate (r_m) at 4.6 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.39	0.00	0.37	0.36	0.33	0.34	0.34	0.39	0.30	0.28	0.30
2	0.32	0.40	0.37	0.31	0.00	0.26	0.34	0.38	0.34	0.35	0.35
3	0.34	0.38	0.32	0.36	0.35	0.35	0.00	0.37	0.32	0.33	0.32
4	0.38	0.35	0.31	0.00	0.32	0.32	0.32	0.39	0.34	0.37	0.35
5	0.38	0.36	0.33		0.35	0.29	0.32	0.38	0.31	0.36	0.00
6	0.35	0.39	0.34		0.31		0.29	0.38	0.33	0.37	0.00

Table S2.12: Values of population growth rate (r_m) at 10 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.36	0.39	0.29	0.34	0.30	0.31	0.40	0.39	0.27	0.32	0.29
2	0.36	0.38	0.31	0.35	0.00	0.32	0.32	0.39	0.30	0.34	0.00
3	0.32	0.35	0.31	0.28	0.29	0.33	0.36	0.37	0.29	0.33	0.31
4	0.32	0.37	0.32	0.00	0.00	0.30	0.36	0.00	0.28	0.00	0.33
5	0.29	0.36	0.26		0.00	0.26	0.28	0.35	0.31	0.33	0.27
6	0.24	0.37	0.32		0.22		0.30	0.36	0.34	0.35	0.26

Table S2.13: Values of population growth rate (r_m) at 22 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.00	0.00	0.24	0.21	0.00	0.00	0.11	0.30	0.00	0.23	0.20
2	0.00	0.27	0.23	0.06	0.00	0.06	0.00	0.23	0.00	0.24	0.00
3	0.00	0.34	0.08	0.00	0.14	0.20	0.00	0.30	0.17	0.19	0.22
4	0.00	0.29	0.24	0.00	0.00	0.17	0.00	0.30	0.25	0.28	0.00
5	0.23	0.39	0.14		0.08	0.22	0.00	0.32	0.00	0.28	0.00
6	0.15	0.29	0.24		0.16		0.00	0.26	0.00	0.27	0.00

Table S2.14: Median genetic variance (V_G), environmental variance (V_E) and phenotypic variance (V_P) for total reproduction (R_0). Numbers between brackets represents the 90% confidence interval.

Cd concentration (µg Cd/L)	V_E	V_G	V_P
0	523.03 [241.35 – 866.85]	300.51 [41.19 – 602.08]	840.25 [486.57 – 1162.26]
1	621.38 [297.48 – 1052.08]	143.01 [1.17 – 306.34]	772.01 [425.63 – 1179.33]
2.2	759.07 [410.42 – 1211.98]	204.04 [0 – 461.57]	987.53 [569.70 -1371.85]
4.6	732.90 [504.98 – 953.94]	441.93 [48.45 – 954.45]	1173.55 [836.95 – 1560.24]
10	510.48 [259.33 – 864.83]	382.43 [0 – 757.92]	893.11 [398.16 – 1469.08]
22	25.03 [15.13 – 36.56]	18.95 [4.11 – 33.72]	45.15 [24.71 – 58.89]

Table S2.15: Median genetic variance (V_G), environmental variance (V_E) and phenotypic variance (V_P) for population growth rate (r_m). Numbers between brackets represents the 90% confidence interval.

Cd concentration (µg Cd/L)	V_E	V_G	V_P
0	6.36E-03 [5.78E-04 - 1.28E-02]	5.07E-04 [0 - 1.18E-04]	6.95E-03 [9.83E-04 - 1.26E-02]
1	7.11E-03 [6.63E-04 - 1.46E-02]	1.16E-03 [0 - 3.89E-04]	8.30E-03 [9.86E-04 - 1.69E-02]
2.2	8.80E-03 [9.20E-04 - 1.82E-02]	2.80E-03 [1.42E-04 - 6.45E-04]	1.20E-02 [1.02E-03 - 2.11E-02]
4.6	1.07E-02 [4.09E-03 - 1.7E-02]	2.70E-04 [0 - 1.92E-03]	1.11E-02 [4.62E-03 - 1.72E-02]
10	9.68E-03 [4.16E-03 - 1.59E-02]	1.85E-03 [0 - 5.85E-03]	1.15E-02 [4.67 E-03 - 1.89 E-02]
22	7.68E-03 [4.65E-03 - 1.11E-02]	7.42E-03 [2.25E-03 - 1.64E-03]	1.51E-02 [9.94E-03 - 1.95E-02]

Supplementary Material S2.1

Bootstrap resampling.

The bootstrap procedure repeatedly draws random samples from the origingal data set with replacement. The number of ways the data set can be sampled is infinite, usually a thousand or more analyses are performed to arrive at stable average values for the parameter estimates and their standard errors. As our interest is in the among-clones variance, bootstrapping would be done over clones (Lynch et al., 1998; pp 570). Suppose the original dataset consists of n individuals. A bootstrap resample is generated drawning n values, with replacement from the original dataset. Such a sample will have some of the original values present multiple times and others not present at all. A series of N such samples are generated and an estimate is computed for each, generating a distribution of estimates.

Example: In chapter 2, a total of 11 clones was used (with 10 replicates) and 5000 bootstrap resamples were used (see Table S2.16). At each bootstrap, the variance between (V_G) and within (V_E) clones was determined.

Table S2.16: Example of first three bootstraps used in Chapter 2. At each bootstrap V_G and V_E are determined.

Bootstrap number	Clone number										
Bootstrap 1	11	9	5	9	10	4	6	10	8	4	11
Boostrap 2	4	6	1	5	5	4	3	8	7	9	1
Bootstrap 3	1	2	11	9	11	3	4	1	10	3	8

Method of moments

Method of moments is a general procedure of estimating variance components from observed mean squares. Table S2.17. Presents the observed mean squares for unequal clones sizes.

Table S1.17: Summary of one-way ANOVA involving N independent clones, the ith wich contains ni replicates.df is degrees of freedom, SS is sums of squares and E(MS) is expected mean squares.

Factor	df	SS	MS	E(MS)
Among clones	N-1	$SS_G = \sum_{i=1}^{N} n_i (z_i - z)^2$	$SS_G/(N-1)$	$\sigma_E^2 + n_0\sigma_G^2$
Within-clones	T-N	$SS_E = \sum_{i=1}^{N}\sum_{j=1}^{n_i} (z_{ij} - z_i)^2$	$SS_E/(T-N)$	σ_E^2
Total	T-1	$SS_T = \sum_{i=1}^{N}\sum_{j=1}^{n_i} (z_{ij} - z)^2$	$SS_T/(T-1)$	σ_t^2

Table S3.1: Results of the preliminary 14-day chronic ecotoxicity test with Cd. Values represent chronic EC_{50} and EC_{10} values (µg Cd/L) for the 3 *D. magna* clones tested. Numbers between brackets represents the 95 % confidence interval.

Clone	EC_{50} [95% C.I.]	EC_{10} [95% C.I.]
1	15.33 [10.76-21.84]	4.81 [2.36-9.85]
2	12.55 [7.59-20.76]	3.16 [1.10-9.00]
3	7.03 [5.8-8.52]	5.30 [4.48-6.37]

Table S3.2: Physico-chemical characteristics of test media during the Cd exposure experiment. Values represent mean ± standard deviation.

	20 °C (Control)		20°C and Cd		24°C (Control)		24°C and Cd	
	New medium	Old medium	New medium	Old medium	New medium	Old medium	New medium	Old medium
Cd (µg Cd/L)	<0.1	<0.1	4.3 ± 0.2	2.9 ± 0.5	<0.1	<0.1	4.3 ± 0.2	2.9 ± 0.7
DOC (mg C/l)	3.7 ± 0.2	5.4 ± 0.4	4.1 ± 0.1	5.0 ± 0.4	3.9 ± 0.2	5.7 ± 0.3	4.1 ± 0.4	5.1 ± 0.3
pH	7.3 ± 0.4	7.6 ± 0.5	7.5 ± 0.3	7.7 ± 0.4	7.5 ± 0.3	7.5 ± 0.4	7.4 ± 0.2	7.7 ± 0.3
Dissolved oxygen (mg O_2/l)	8.9	8.7 ± 0.3	8.7	9.0 ± 0.4	8.5	7.6 ± 0.3	8.6	8.1 ± 0.4

Table S3.3: Values of reproduction during 21 days at 20°C.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	92	117	99	88	106	134	86	100	139	148	104	102	112	79
2	91	97	98	128	114	150	98	76	131	152	92	86	86	90
3	94	99	85	112	103	135	102	102	124	204	117	168	92	103
4	99	112	109	110	117	133	129	103	115	186	138	124	119	95
5	72	104	98	98	102	138	131	104	119	194	127	136	113	85
6	85	101	98	128	110	150	101	102	122	180	139	133	117	100
7		95	103	129	124	136	104	78	141	178	68	80	90	91
8		88	105		133	98	102	119	125	162	82		107	97
9		109	93		106		94	77		125	136		83	
10		109			93		88			123	118			

Table S3.4: Values of r_m at 20°C.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0.38	0.37	0.43	0.40	0.40	0.44	0.37	0.37	0.44	0.38	0.38	0.40	0.42	0.35
2	0.41	0.35	0.41	0.39	0.37	0.45	0.39	0.37	0.44	0.39	0.39	0.37	0.40	0.39
3	0.42	0.36	0.42	0.42	0.40	0.45	0.40	0.41	0.41	0.41	0.42	0.42	0.43	0.38
4	0.42	0.37	0.41	0.38	0.42	0.43	0.40	0.42	0.44	0.40	0.42	0.44	0.43	0.41
5	0.38	0.35	0.39	0.41	0.39	0.44	0.41	0.41	0.42	0.41	0.42	0.43	0.43	0.36
6	0.39	0.37	0.42	0.42	0.43	0.46	0.40	0.42	0.43	0.38	0.44	0.44	0.43	0.41
7		0.37	0.42	0.43	0.45	0.41	0.41	0.38	0.43	0.39	0.41	0.42	0.44	0.38
8		0.37	0.40		0.45	0.39	0.41	0.41	0.45	0.39	0.38		0.40	0.36
9		0.38	0.38		0.37		0.37	0.43		0.39	0.43		0.44	
10		0.35			0.39		0.37			0.40	0.43			

Table S3.5: Values of reproduction at first brood at 20°C.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	14	13	15	10	16	16	13	12	14	14	12	16	18	16
2	13	8	14	11	12	18	14	13	16	15	16	12	16	15
3	14	9	13	13	10	19	15	12	11	15	12	23	15	21
4	13	11	13	8	11	15	13	14	14	13	11	16	15	21
5	16	10	12	11	11	16	16	11	13	15	14	16	16	12
6	16	11	14	13	10	20	15	14	14	10	15	17	16	20
7		12	13	14	14	13	17	16	14	12	13	14	15	14
8		12	14		14	15	15	12	19	11	13		16	15
9		11	13		8		11	16		14	12		17	
10		12			16		12			14	15			

Table S3.6: Values of size at day 21 at 20°C.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	4	4.34	4.11	4.35	3.96	4.17	4.07	4.18	4.36	3.97	4.39	4.56	4.19	3.87
2	4.14	4.35	3.92	4.32	3.93	4.62	4.06	4.25	4.32	3.84	4.13	4.63	4.2	4.21
3	3.87	4.16	4.35	4.41	4.05	4.19	4.13	4.38	4.18	4.21	4.28	4.45	4.28	4.23
4	4.11	4.06	3.88	4.18	4.02	4.23	3.93	4.26	4.36	3.92	4.14	4.75	4.05	4.2
5	3.74	4.15	3.83	4.51	4.08	4.03	3.98	4.11	4.27	3.91	4.33	4.41	4.21	4.08
6	3.71	4.25	3.78	4.54	3.84	4.03	4.06	4.24	4.15	4.07	4.23	4.56	4.36	4.22
7		4.18	4.18	4.15	3.9	4.06	4.15	4.26	4.15	4.08	3.77	4.3	4.18	4.07
8		4.34	4.31		4.07	4.04	3.87	4.33	4.24	4.04	4.07		4.22	4.19
9		4.2	3.85		4.22		4.33	4.21		3.81	4.3		4.25	
10		4.43			4.13		3.88			4.1	3.97			

Table S3.7: Values of length at first brood at 20°C.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	3.35	3.07	2.89	3.00	3.14	3.30	3.23	3.22	3.17	3.25	3.22	3.25	3.14	3.38
2	2.70	2.91	3.01	3.08	3.06	2.98	2.98	3.12	3.03	2.96	3.38	3.36	3.18	2.86
3	2.94	3.06	3.20	3.33	2.78	3.28	3.13	3.02	3.22	3.08	3.06	3.44	3.33	3.47
4	2.80	2.91	3.00	3.10	2.95	3.28	2.98	3.25	3.16	3.12	3.09	3.06	3.04	3.12
5	3.26	2.84	2.99	3.17	2.85	3.19	3.11	2.89	3.04	2.88	3.04	3.26	3.26	3.24
6	3.12	3.13	3.01	2.94	2.94	3.19	2.98	2.92	3.03	3.04	3.01	3.04	3.40	3.00
7		3.14	2.79	2.99	3.09	3.30	3.10	3.03	3.20	2.93	2.85	3.02	3.15	3.24
8		3.10	3.06		3.17	3.47	2.97	3.09	3.15	3.00	3.32		3.25	3.08
9		3.17	3.23		2.79		3.15	3.08		2.97	3.20		3.32	
10		3.08			2.98		3.08			3.17	2.98			

Table S3.8: Values of time to first brood at 20°C.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	8	8	7	7	8	7	8	8	7	8	8	8	8	8
2	7	8	7	7	8	7	8	8	7	8	8	8	8	7
3	7	8	7	7	7	7	8	7	7	8	7	8	7	8
4	7	8	7	7	7	7	8	7	7	8	7	7	7	7
5	8	8	7	7	7	7	8	7	7	8	7	7	7	7
6	8	8	7	7	7	7	8	7	7	8	7	7	7	7
7		8	7	7	7	8	8	7	7	8	7	7	8	7
8		8	8		7	8	8	7	7	8	8		7	8
9		8	8		7		8	7		8	7		8	
10		9			8		8			8	7			

Table S3.9: Values of reproduction during 21 days at 20°C and Cd.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	96	116	88	85	124	67	96	91	87	89	101	133	92	109
2	117	91	112	106	132	66	99	95	152	76	107	101	109	95
3	112	85	106	117	117	59	85	99	141	74	126	88	111	80
4	97	137	134	92	117	51	113	111	125	87	132	121	111	83
5	113	87	110	101	105	51	86	96	91	77	70	135	106	98
6	109	81	115	104	98	61	120	102	120	76	77	87	84	92
7	81	67	126	105	113	57	130	106	77	66	111	96	112	88
8	90		96	108	104	47	92	92	83		136	99		92
9			124		104	80	96	69	71		139	99		82
10						62	93		114					

Table S3.10: Values of r_m at 20°C and Cd.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0.42	0.40	0.40	0.42	0.41	0.42	0.37	0.41	0.42	0.35	0.39	0.37	0.37	0.40
2	0.43	0.37	0.44	0.35	0.38	0.42	0.37	0.43	0.45	0.35	0.41	0.43	0.38	0.39
3	0.42	0.36	0.41	0.40	0.41	0.45	0.36	0.43	0.42	0.35	0.42	0.41	0.41	0.34
4	0.39	0.40	0.44	0.44	0.43	0.43	0.38	0.40	0.41	0.34	0.44	0.43	0.39	0.38
5	0.43	0.39	0.42	0.42	0.36	0.42	0.36	0.43	0.44	0.33	0.37	0.44	0.36	0.40
6	0.42	0.37	0.45	0.42	0.37	0.45	0.37	0.42	0.42	0.34	0.38	0.41	0.37	0.39
7	0.39	0.38	0.41	0.42	0.40	0.39	0.39	0.43	0.43	0.34	0.41	0.40	0.40	0.39
8	0.37		0.42	0.41	0.42	0.42	0.37	0.43	0.44		0.44	0.40		0.35
9			0.44		0.39	0.44	0.37	0.38	0.44		0.44	0.38		0.35
10						0.41	0.37		0.45					

Table S3.11: Values of reproduction at first brood at 20°C and Cd

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	14	16	16	12	10	18	12	7	11	12	14	15	9	17
2	14	12	17	10	11	14	11	13	15	11	14	14	8	15
3	13	9	12	11	10	17	11	13	11	11	13	17	11	12
4	9	13	16	15	10	14	12	6	9	9	15	13	9	14
5	14	10	14	14	9	12	11	10	13	7	12	15	8	19
6	12	10	16	12	16	17	9	10	11	9	12	15	10	16
7	13	11	14	12	5	10	14	12	12	8	15	12	13	16
8	6		15	10	10	13	10	14	14		16	17		13
9			16		15	14	11	7	13		15	9		12
10						13	11		15					

Table S3.12: Values of size at day 21 at 20°C and Cd.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	3.69	4.05	4.13	3.95	3.95	4.03	4.11	3.7	3.79	3.94	4.27	4.4	4.11	3.88
2	3.91	4.06	4.08	4.25	3.98	4.01	3.89	3.59	4.29	3.89	3.94	4.35	3.57	4.03
3	4.07	3.73	4.03	4.04	4.09	4.19	3.83	4.14	4.3	4.01	4.18	4.45	3.97	3.97
4	3.85	3.75	4.28	4.35	3.96	4.04	3.83	4	4.13	3.95	3.97	4.32	3.94	3.84
5	3.97	3.39	4.13	4.3	4.2	4.25	3.88	4.03	4.2	3.85	4.06	4.63	4.03	4.02
6	3.58	3.62	4.16	4.47	4.23	4.41	4.1	4.28	4.12	3.66	4.02	4.38	4	4.11
7	3.75	3.77	4	4.64	4.09	4.01	4.14	4.11	3.92	3.81	3.96	4.54	3.93	3.61
8	3.76		4.06	4.04	4	4.05	4.01	3.99	3.95		4.24	4.15		3.78
9			3.95		4.11	4.02	3.98	3.95	4.18		4.08	4.31		3.78
10						4.12	4.1		4.57					

Table S3.13: Values of length at first brood at 20°C and Cd.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	2.89	3.29	3.03	3.04	3.01	3.43	3.15	3.04	3	2.98	2.99	3.21	3.17	3.15
2	2.76	3.06	3.02	3.1	3.13	3.35	3.2	3.02	3.09	2.95	3.13	3.22	3.12	3.04
3	2.96	3.07	3.01	3.13	3.17	3.03	3.02	3.05	2.99	3.05	2.99	3.3	3.1	2.91
4	3.17	3.21	3.1	3.17	2.94	3.39	3.02	2.82	2.84	3.01	3.09	3.24	3.09	3.04
5	2.86	3.07	2.96	2.92	2.84	3.33	2.83	2.74	3.06	2.9	3.13	3.1	3.1	3.1
6	2.89	2.82	3.13	2.99	3.14	3.41	3.04	2.76	3.12	2.89	2.98	3.33	3.07	3.13
7	3	3.07	2.96	2.98	2.88	3.2	3.06	3.01	3.14	2.88	3.22	3.31	2.95	2.95
8	2.92		2.96	3.15	2.92	3.17	2.9	3.01	3.11		3.28	3.51		2.75
9			2.96		3.06	2.95	2.97	2.75	3.1		3.09	3.13		3.07
10						3.41	2.96		3.15					

Table S3.14: Values of time to first brood at 20°C and Cd.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	7	8	8	7	7	8	8	8	7	9	8	8	8	8
2	7	8	7	10	8	8	8	8	7	9	8	7	7	8
3	7	8	7	7	7	7	8	8	7	9	7	8	7	9
4	7	8	7	7	7	7	8	8	7	9	7	7	7	8
5	7	8	7	7	8	7	8	8	7	9	8	7	8	8
6	7	8	7	7	9	7	8	8	7	9	7	8	8	8
7	8	8	8	7	7	8	8	8	7	9	7	8	8	8
8	7		7	7	7	7	8	8	7		7	8		9
9			7		8	7	8	8	7		7	8		9
10						8	8		7					

Table S3.15: Values of Reproduction during 21 days at 24°C.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	105	130	118	124	136	119	128	123	114	118	101	138	134	88
2	110	156	116	125	158	126	119	115	103	96	127	131	126	99
3	112	126	107	126	177	104	140	114	122	144	105	123	131	99
4	91	142	113	107	162	107	112	109	121	120	186	118	138	91
5	109	170	123	112	160	151	119	105	105	94	158	70	137	94
6	87	149	101	97		75	118	120	107	116	151	107	140	89
7	103	136	109	90		106	112	110	86	127	177	110	123	110
8	88	169	105			106	119	118	107	100	149	112		
9		125	105					117	121	100	151	100		
10		155						115	112			107		

Table S3.16: Values of r_m at 24°C.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0.39	0.43	0.48	0.46	0.48	0.47	0.42	0.45	0.47	0.52	0.47	0.48	0.44	0.44
2	0.44	0.44	0.42	0.49	0.42	0.48	0.40	0.45	0.47	0.49	0.45	0.48	0.43	0.45
3	0.45	0.41	0.48	0.48	0.49	0.44	0.45	0.52	0.49	0.45	0.47	0.46	0.42	0.45
4	0.46	0.44	0.42	0.49	0.54	0.45	0.47	0.46	0.49	0.52	0.50	0.46	0.41	0.44
5	0.41	0.42	0.43	0.50	0.47	0.47	0.45	0.51	0.51	0.49	0.56	0.47	0.40	0.44
6	0.41	0.43	0.46	0.31		0.47	0.43	0.49	0.51	0.47	0.48	0.46	0.44	0.41
7	0.46	0.47	0.42	0.45		0.43	0.47	0.47	0.51	0.51	0.49	0.45	0.42	0.40
8	0.41	0.47	0.42			0.45	0.43	0.44	0.52	0.51	0.51	0.47		
9		0.43	0.43					0.51	0.50	0.46	0.48	0.46		
10		0.42						0.50	0.49			0.45		

Table S3.17: Values of reproduction at first brood at 24°C.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	10	11	14	9	9	21	13	18	10	16	13	13	8	10
2	9	10	12	13	14	12	13	18	11	11	17	8	7	11
3	12	8	14	11	9	10	15	14	14	16	12	11	6	11
4	12	13	12	12	9	19	10	10	13	14	11	10	7	10
5	7	8	14	13	20	19	16	13	14	10	12	11	7	10
6	11	8	12	8		10	14	11	12	11	8	11	7	7
7	12	10	11	19		16	13	11	14	14	9	9	10	17
8	15	10	12			12	14	16	15	13	7	11		
9		10	14					13	10	10	9	10		
10		9						12	14			10		

Table S3.18: Values of size at day 21 at 24°C.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	3.93	4.31	4.17	4.14	4.28	4.23	4.14	4.28	4.25	4.04	4.09	4.34	3.99	3.86
2	4.04	426	3.99	3.94	4.06	3.89	3.95	4.08	4.16	4.2	4.15	3.93	4.37	3.89
3	4.08	4.56	4.03	4.12	4.1	4.3	4.02	4	3.85	4.17	4.15	4.25	4.43	4.29
4	3.73	4.32	4.39	4.27	4.07	4.17	4.06	4.06	4.09	4.24	4.45	4.73	4.38	3.96
5	4.33	3.97	4.19	3.98	3.96	3.97	3.89	4.04	4.14	3.96	4.13	4.18	4.31	4.07
6	3.98	3.97	4.14	3.99		4.18	4.19	4.34	4.16	4	4.06	4.27	3.88	3.82
7	4.22	4.18	4.28	3.91		4.22	3.71	4.15	4.25	4.29	4.1	4.57	4.28	3.75
8	3.96	3.91	4.07			4.29	3.8	4.17	4.37	3.98	4.39	4.54		
9		4.19	4.09					4.17	4	3.9	4.41	4.52		
10		4.36						3.94	3.94			3.5		

Table S3.19: Values of length at first brood at 24°C.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	2.98	2.85	3.29	2.96	2.86	3.44	2.97	3.11	3.05	3.03	2.88	3.19	3.16	2.93
2	3.15	3.03	2.91	2.88	3	2.96	2.73	3.03	2.98	2.9	2.99	2.92	3	2.84
3	3.05	2.91	3.01	2.97	2.91	2.96	2.94	2.9	2.94	2.87	3.21	2.88	2.79	3.09
4	3.2	2.97	3.02	3.02	2.9	3.1	2.53	2.87	3.03	3.07	2.74	2.9	3	2.79
5	2.92	2.87	2.65	2.96	3.18	3.32	2.8	2.9	3.04	3.02	3.06	3.02	2.87	2.85
6	3.11	2.98	3.1	3.28		3.15	3.14	3	2.98	3.12	3.14	3.21	3.1	2.89
7	3.05	2.87	2.75	3.12		3.17	2.64	2.91	2.76	2.89	3	3.11	2.88	2.84
8	2.96	2.85	2.91			2.81	2.88	2.95	2.95	3.06	2.94	3.07		
9		2.83	2.97					2.91	2.92	2.84	3.08	3.04		
10		2.88						2.92	2.85			2.91		

Table S3.20: Values of time to first brood at 24°C.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	7	7	6	6	6	7	7	7	6	6	6	6	6	6
2	6	7	7	6	8	6	8	7	6	6	7	6	6	6
3	6	7	6	6	6	6	7	6	6	7	6	6	6	6
4	6	7	7	6	6	7	6	6	6	6	6	6	7	6
5	6	7	7	6	7	7	7	6	6	6	5	6	7	6
6	7	7	6	10		6	7	6	6	6	6	6	6	6
7	6	6	7	7		7	6	6	6	6	6	6	7	8
8	7	6	7			6	7	7	6	6	5	6		
9		7	7					6	6	6	6	6		
10		7						6	6			6		

Table S3.21: Values of Reproduction during 21 days at 24°C and Cd.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	73	119	97	106	106	67	111	103	109	113	29	138	91	76
2	86	122	104	118	106	99	101	77	100	102	70	133	115	70
3	97	95	110	108	133	27	106	93	107	107	110	157	83	88
4	70	124	94	112	114	58	108	77	108	106	46	141	95	73
5	81	100	101	111	104	87	96	97	101	110	79	119	122	78
6	90	75	132	109		44	107	83	99	69	11	125	123	51
7	84	120	95	108		31	102	99	78	104	90	125	103	77
8	79	102	105	125		81	112	46	97	73	59	137	106	
9	83			138		82	100	88	106	109			111	
10						54	90	89	107	104				

Table S3.22: Values of rm at 24°C and Cd.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0.42	0.44	0..45	0.47	0.43	0.48	0.43	0.48	0.43	0.44	0.42	0.41	0.39	0.41
2	0.45	0.42	0.46	0.47	0.42	0.45	0.40	0.46	0.46	0.46	0.43	0.41	0.38	0.35
3	0.48	0.45	0.47	0.45	0.45	0.42	0.43	0.50	0.46	0.39	0.45	0.44	0.41	0.40
4	0.45	0.42	0.42	0.48	0.43	0.46	0.42	0.47	0.46	0.43	0.43	0.42	0.40	0.39
5	0.43	0.44	0.43	0.49	0.43	0.47	0.40	0.48	0.44	0.46	0.41	0.39	0.45	0.41
6	0.43	0.42	0.43	0.48		0.43	0.40	0.47	0.45	0.43	0.37	0.39	0.46	0.37
7	0.42	0.48	0.41	0.43		0.39	0.43	0.40	0.45	0.39	0.39	0.39	0.42	0.42
8	0.43	0.45	0.46	0.47		0.44	0.43	0.50	0.45	0.41	0.48	0.41	0.42	
9	0.42			0.49		0.46	0.43	0.39	0.47	0.40			0.38	
10						0.47	0.41	0.46	0.41	0.43				

Table S3.23: Values of reproduction at first brood at 24°C and Cd.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	7	8	11	10	8	13	12	9	11	8	11	10	8	7
2	9	9	12	9	7	10	8	7	9	9	11	9	9	4
3	10	8	12	9	16	14	12	8	10	10	9	10	7	6
4	8	9	12	11	8	9	11	9	10	9	11	11	8	5
5	7	8	8	12	7	11	7	10	7	11	9	9	9	6
6	10	7	15	12		16	6	9	9	9	9	10	10	5
7	12	8	9	9		12	12	10	9	10	9	9	7	8
8	9	5	12	10		9	7	12	11	9	11	10	8	
9	6			11		12	13	11	11	11			9	
10						13	9	8	9	9				

Table S3.24: Values of size at day 21 at 24°C and Cd.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	3.54	4.14	4.04	3.9	3.69	4.4	3.53	4.02	4.13	4.06	3.67	4.35	4.03	3.63
2	3.8	3.77	3.94	3.85	3.79	3.84	3.73	4.06	4.03	3.57	3.97	4.33	4.59	3.56
3	3.72	3.99	3.66	3.8	4.04	4.12	3.73	3.94	4.12	3.95	4.04	4.54	4.42	3.72
4	3.51	3.96	3.72	3.77	3.84	3.74	3.8	3.85	3.85	3.91	4.08	4.21	4.23	3.55
5	3.67	3.63	3.85	4.03	3.63	4.03	4	4.03	4	3.85	3.8	4.14	4.21	3.71
6	3.64	3.92	4.08	4.11		3.62	3.94	3.96	4.16	3.73	3.49	4.38	4.32	3.46
7	3.4	4.03	3.91	3.88		3.96	3.87	3.91	3.81	4.03	3.64	4.47	4.1	3.67
8	3.78	3.75	3.79	4.03		4.02	3.83	3.94	4.18	3.92	3.92	4.28	4.19	
9	3.75			3.85		4	3.93	3.93	4.19	3.99			4.01	
10						4.21	3.57	3.85	3.89	3.99				

Table S3.25: Values of length at first brood at 24°C and Cd.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	2.88	2.76	2.91	2.87	2.64	2.81	2.76	2.75	2.87	2.73	2.91	3	2.86	2.74
2	2.7	2.66	2.95	3.07	2.95	2.89	2.8	2.8	2.88	2.92	2.96	3.07	3.04	2.84
3	2.81	2.79	3	3.08	2.61	3.07	2.89	2.73	2.85	3.02	2.97	3.03	2.93	2.64
4	2.91	2.96	2.8	3.1	3.38	3	2.94	2.8	2.91	2.68	2.88	2.98	2.97	2.68
5	2.8	2.81	2.99	2.91	2.68	2.79	2.8	3.05	2.82	2.99	2.89	2.97	2.92	2.69
6	2.77	2.8	3.02	2.91		3.28	2.92	2.96	3	2.82	3.19	3.05	3.07	2.51
7	2.82	2.65	2.82	2.98		3.13	2.88	2.68	2.98	2.78	2.74	2.9	2.99	2.68
8	2.89	2.74	2.93	2.85		3	2.88	2.82	2.57	2.68	3.15	2.84	2.91	
9	2.74			3.01		2.95	2.83	2.7	2.5	2.76			3.11	
10						3	2.68	2.6	2.93	2.64				

Table S3.26: Values of time to first brood at 24°C and Cd.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	6	6	6	6	6	6	7	6	7	6	6	7	7	6
2	6	7	6	6	6	6	7	6	6	6	6	7	7	6
3	6	6	6	6	7	7	7	6	6	7	6	7	7	6
4	6	7	6	6	6	6	7	6	6	6	6	7	6	6
5	6	6	6	6	6	6	7	6	6	6	6	7	6	6
6	6	6	7	6		7	7	6	6	6	6	7	6	6
7	7	6	7	6		7	7	7	6	7	6	7	6	6
8	6	6	6	6		6	6	6	6	7	6	7	6	
9	6			6		6	7	7	6	7			7	
10						6	7	6	7	6				

Table S3.27: 5th and 95th genetic correlation coefficient between environments of r_m The ρ-values of 95 pt and 5 pt are given left and right of the diagonal, respectively.

		Genetic correlation coefficient – 5pt			
		T20	T20Cd	T24	T24Cd
Genetic correlation coefficient – 95pt	T20		0.41	-0.15	-0.30
	T20Cd	0.98		-0.42	0.37
	T24	0.81	0.85		-0.18
	T24Cd	0.74	0.89	0.69	

Supplementary material S3.28

Power analysis for comparing between-trait genetic correlations among environments

We calculated the statistical power for detecting a significant difference of a between-trait genetic correlation ($\rho_{trait1,trait2}$) among two environments as follows. All calculations and calculations described below were programmed in MATLAB.

We randomly sampled N clone means for traits 1 and 2 in both environments from a multivariate normal distribution using the observed population means of both traits in both environments (see Table 1 of main text), the observed genetic variances of both traits in both environments (see Figure 1 of main text), and the observed 4x4 matrix of Pearson product-moment correlations between clone means (among both traits and both environments, data not shown) as the parameters. The correlated sampling was performed using the Gaussian copula function (Nelsen RB, 1999, An introduction to copulas. Springer, New York, 216p.) in MATLAB (*copularnd*).

In the next step, 10 random samples, representing the individual daphnids of a given clone, were drawn from a normal distribution with the simulated clone mean (obtained in the previous step) and the observed residual variance (data not shown) as the parameters. This step was performed for all N clones, for both traits and for both environments. As such a randomly sampled dataset of trait values was obtained with dimensions 2 (traits) x 2 (environments) x N (clones) x 10 (individuals). This simulated dataset was the input to the same bootstrapping procedure for calculating $\rho_{trait1,trait2}$ for the two environments and the same statistical analysis for comparing these among those environments (see 2.4 in main text). The outcome of this analysis (statistically different or not) was stored and the whole procedure was repeated 100 times. The number of calculations in which a statistically significant difference (p=0.05) was observed was divided by 100 to yield the power.

This entire procedure was performed for all possible between-trait correlations (with 6 traits, this gives 15 correlations in total) and comparisons of $\rho_{trait1,trait2}$ were made between the control and Cd treatment. This was performed for both 20°C and 24°C. Calculations were done for N= 14, 30, 45, 60, 75, 90, 120, 150, and 200. Thus, we obtained a total of 30 power-curves (ρ vs. N) that are representative for the 6 traits investigated in our study (Figure S3.1). S.

At N=14, which was the number of clones used in our present study, the highest power observed was as low as 0.27 (Figure S1). The commonly used design target of a power of b=0.8 is only reached for N = 60 or higher. Obviously, the exact sample size required to reach b=0.8 is also dependent on the absolute difference in $\rho_{trait1,trait2}$ values between the two environments, i.e. abs($\rho_{trait1,trait2,control}$ - $\rho_{trait1,trait2,Cd}$). Indeed for any given N, ρ increases with increasing absolute difference in $\rho_{trait1,trait2}$: the power to detect a statistically significant difference increases with increasing difference in $\rho_{trait1,trait2}$ values. Figure S2, based on the same simulated data as presented in Figure S1, illustrates this for N=14, N=45 and N=200 (plots for other N not shown).

Performing breakpoint linear regression (Figure S2) on these plots made for all N yields the absolute difference in $\rho_{trait1,trait2}$ values between the two environments that can be detected with a typical target power of $\rho = 0.8$ as a function of N (Figure S3). This Figure indicates for example that with a sample size of N= 60 clones, one has a probability of 80% (b=0.8) to detect an absolute difference in $\rho_{trait1,trait2}$ values of 0.68 as a statistically significant difference. At a sample size of N=200 clones absolute differences in $\rho_{trait1,trait2}$ values down to 0.32 can be detected.

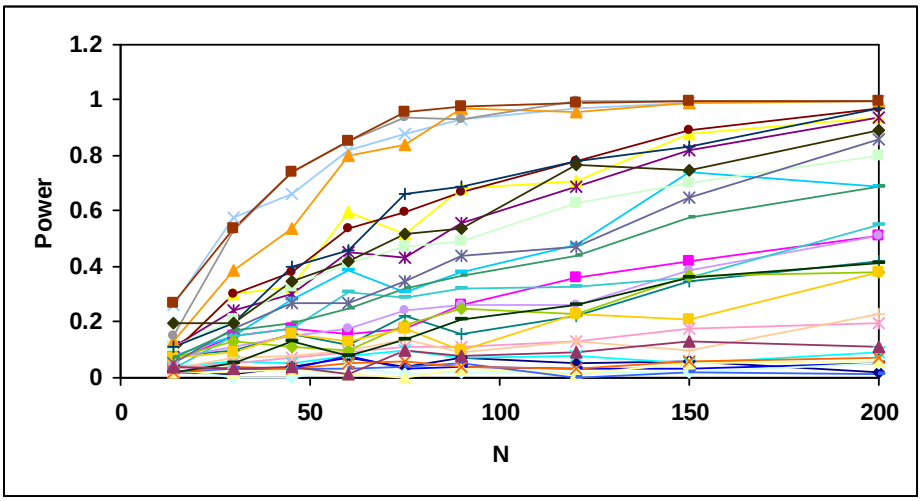

Figure S3.1: Statistical power to detect significant differences of between-trait correlation among two environments as a function of N. Different curves represent calculations for the 30 between-trait correlations investigated in our study.

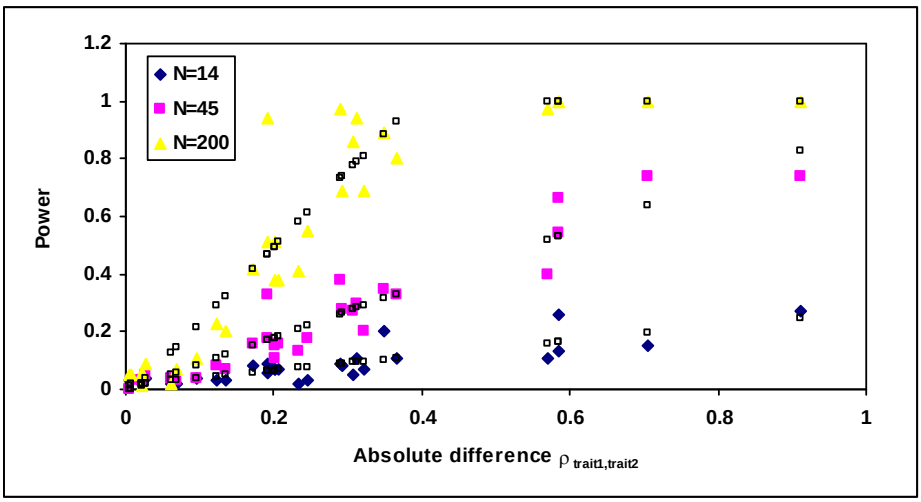

Figure S3.2: Statistical power to detect significant differences as a function of the absolute difference of the between-trait correlation among the two environments. Black empty squares are fitted breakpoint linear regressions.

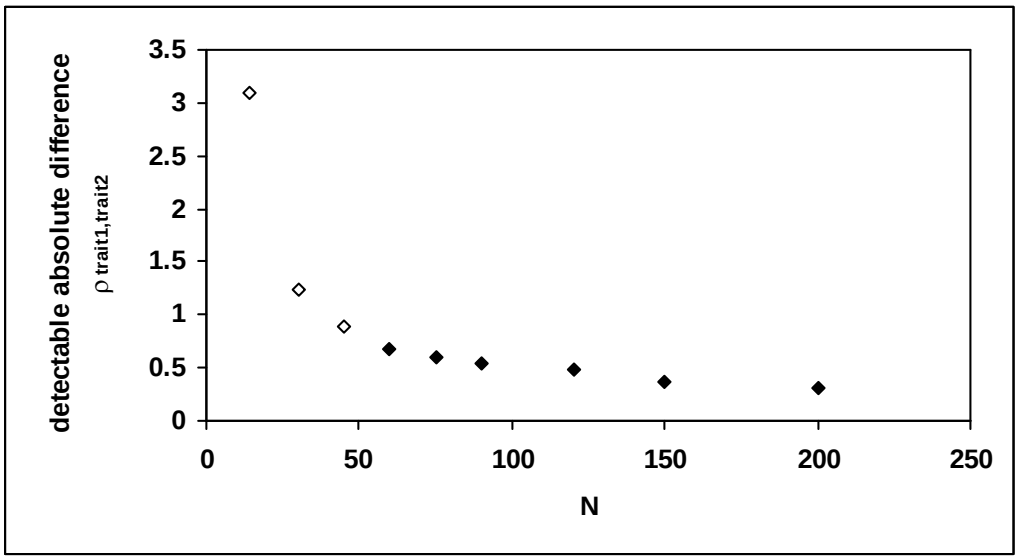

Figure S3.3: The absolute difference of between-trait correlation among two environments that can be detected as significant with a power = 0.8. Empty diamonds are for all N for which none of the 30 calculations yielded a power b equal to or higher than 0.8.

Table S4.1: Physico-chemical characteristics of test media during the Cd exposure experiment. Values represent mean ± standard deviation. NM is new medium. OM is old medium.

Nominal Cd concentration (µg Cd/L)		DOC (mg C/L)	pH	Cd concentration (µg Cd/L)	Mean Cd concentration (µg Cd/L)
0	NM	4.68		< D.L.	0.00
	OM	5.33 ± 0.42	7.47 ± 0.13	< D.L.	
5	NM	4.7		4.52 ± 0.07	3.64
	OM	5.62 ± 0.41	7.53 ± 0.17	2.76 ± 0.03	
0	NM	4.62		< D.L.	0.00
	OM	5.41 ± 0.51	7.46 ± 0.17	< D.L.	
5	NM	4.58		4.56 ± 0.08	3.81
	OM	5.41 ± 0.38	7.61 ± 0.15	3.05 ± 0.08	

Table S4.2: Values of total reproduction at 20°C.

Clone / Replicate	A	B	C	D	F	G	H	J	K	N
1	93	117	61	116	113	137	68	126	72	92
2		127	33	132	115	105	96	120	104	78
3	92	111	161	24	105	108	67	110	152	78

Table S4.3: Values of total reproduction at 20°C.

Clone / Replicate	O	P	R	S	U	V	W	Y	E	I
1	136	88	135	141	149	48	211	124	125	
2	27	37		122	179	97	182	142	136	
3		58		75		58		121	143	

Table S4.4: Values of total reproduction at 20°C.

Clone / Replicate	CW	EF	EY	RK	IH	NK	GV	RD	ID	FN
1	109	71	46	110		108	116		140	126
2	111	78		0	140	127	109		168	114
3	119	60		79		131	117	109	114	113

Table S4.5: Values of total reproduction at 20°C.

Clone / Replicate	SY	AW	DU	HU	YW	IS	EW	CR	RU	RJ
1	139	84	134	117	25	114		158	168	151
2	0		111	135	113	0	114	151	128	137
3	96	80	112	99	98	95		151	148	118

Table S4.6: Values of total reproduction at 20°C.

Clone / Replicate	BA	OA	GW	KE	PV	JH	JY	EN	VF	CE
1		84	66	135	60	116		29	125	166
2		61	135	104	52	87	145	14	104	113
3		34	0	127	67	101	118	2	112	92

Table S4.7: Values of total reproduction at 20°C.

Clone / Replicate	FY	FD	SP	HR	SD	NA	RN	DA	NU
1	121	134	119	146	132	96	129	117	127
2			124	152	133		118	115	145
3		102	118	43	131	97	189	121	

Table S4.8: Values of total reproduction at 20°C +Cd.

Clone / Replicate	A	B	C	D	F	G	H	J	K	N
1	84	90	83	123	111	120	93	98	61	102
2		87	82	89	107	89	94	103	95	84
3	84	70	44	103	78	99	103	110	89	113

Table S4.9: Values of total reproduction at 20°C +Cd.

Clone / Replicate	O	P	R	S	U	V	W	Y	E	I
1	103	72	48	97	123	88	121	108	60	
2	124	72		107	155	82	146	128	46	
3		33		117		81	21	126	57	

Table S4.10: Values of total reproduction at 20°C +Cd.

Clone / Replicate	CW	EF	EY	RK	IH	NK	GV	RD	ID	FN
1	100	87	0			93	89		0	61
2	74	98		42	16	67	72		0	0
3	105	85		49		95	54	83	126	66

Table S4.11: Values of total reproduction at 20°C +Cd.

Clone / Replicate	SY	AW	DU	HU	YW	IS	EW	CR	RU	RJ
1	125	81	100	0	116	77		90	120	88
2	0		116	102	123	68		93	111	87
3	115		91	87	94	4		106	92	85

Table S4.12: Values of total reproduction at 20°C +Cd.

Clone / Replicate	BA	OA	GW	KE	PV	JH	JY	EN	VF	CE
1		52	97	68	90	113		28	61	117
2		55	92	103	82	83	83	27	78	101
3		61	0	103	64	80	81	6	90	0

Table S4.13: Values of total reproduction at 20°C +Cd.

Clone / Replicate	FY	FD	SP	HR	SD	NA	RN	DA	NU
1	107	92	90	101	140	82	72	48	142
2			91	107	129	95	111	31	96
3		29	137	123	119	85	114	31	

Table S4.14: Values of total reproduction at 24°C.

Clone / Replicate	A	B	C	D	F	G	H	J	K	N
1	144	109	76	144	136	110	156	120	148	159
2	140	103	91	171	24	111	135	109	138	161
3	16		152	179	114	101	154	114	161	151

Table S4.15: Values of total reproduction at 24°C.

Clone / Replicate	O	P	R	S	U	V	W	Y	E	I
1	183	109	30	129	105	112	148	136	120	
2	175	98	86	131	90	92	115	169	138	
3	84	95	43	124	156	80		164	125	

Table S4.16: Values of total reproduction at 24°C.

Clone / Replicate	CW	EF	EY	RK	IH	NK	GV	RD	ID	FN
1	86	94		72	165	99	89	206	0	67
2	133	76		98	161	100	117	0	75	76
3	162	77		76	0	67	102	116	0	

Table S4.17: Values of total reproduction at 24°C.

Clone / Replicate	SY	AW	DU	HU	YW	IS	EW	CR	RU	RJ
1	126	70	99	73	119	0	137	155	120	148
2	0	106	147	91	49		141	0	119	152
3	132	80	177	164	121		97	142	122	0

Table S4.18: Values of total reproduction at 24°C.

Clone / Replicate	BA	OA	GW	KE	PV	JH	JY	EN	VF	CE
1	23	6	154	141	72	0	93	0	98	21
2	63	0	91	75	106	137	162	5	97	81
3		42		75	51	103	115		33	66

Table S4.19: Values of total reproduction at 24°C.

Clone / Replicate	FY	FD	SP	HR	SD	NA	RN	DA	NU
1	144	98	135	213	147	35	47	126	72
2	125	102	93	93	127	36	102	142	86
3	135	110		148	107	102	148	148	22

Table S4.20: Values of total reproduction at 24°C and Cd.

Clone / Replicate	A	B	C	D	F	G	H	J	K	N
1	7	1	0	18	7	0	13	102	8	9
2	5	16	0	12	10	0	22	39	0	4
3	28		89	0	10	0	10	33	0	0

Table S4.21: Values of total reproduction at 24°C and Cd.

Clone / Replicate	O	P	R	S	U	V	W	Y	E	I
1	0	4	0	4	7	0	3	12	3	
2	0	4	0	6	8	6	0	0	6	
3	0	3	0	0	8	0		12	4	

Table S4.22: Values of total reproduction at 24°C and Cd.

Clone / Replicate	CW	EF	EY	RK	IH	NK	GV	RD	ID	FN
1	0	0		0	0	6	7	8	0	28
2	2	5		5	0	0	6	38	43	19
3	0	0		14	0	14	1	3	0	

Table S4.23: Values of total reproduction at 24°C and Cd.

Clone / Replicate	SY	AW	DU	HU	YW	IS	EW	CR	RU	RJ
1	0	0	7	0	10	0	8	13	35	7
2	0	48	14	0	0		6	10	31	45
3	2	78	29	5	0		4	4	5	55

Table S4.24: Values of total reproduction at 24°C and Cd.

Clone / Replicate	BA	OA	GW	KE	PV	JH	JY	EN	VF	CE
1	32	21	3	6	14	74	0	0	15	18
2	12	68	0	7	3	71	3	0	28	20
3		0		8	62	58	1		6	0

Table S4.25: Values of total reproduction at 24°C and Cd.

Clone / Replicate	FY	FD	SP	HR	SD	NA	RN	DA	NU
1	10	26	2	7	65	0	8	0	5
2	0	15	0	10	36	6	0	0	13
3	0	0		6	0	4	0	0	3

Table S4.26: Values of population growth rate at 20°C.

Clone / Replicate	A	B	C	D	F	G	H	J	K	N
1	0.36	0.34	0.34	0.38	0.35	0.35	0.33	0.38	0.36	0.39
2		0.31	0.31	0.39	0.34	0.37	0.35	0.35	0.43	0.35
3	0.36	0.29	0.42	0.33	0.35	0.36	0.34	0.35	0.43	0.36

Table S4.27: Values of population growth rate at 20°C.

Clone / Replicate	O	P	R	S	U	V	W	Y	E	I
1	0.46	0.40	0.44	0.53	0.47	0.41	0.48	0.43	0.46	
2	0.40	0.35		0.50	0.45	0.36	0.45	0.40	0.46	
3		0.39		0.39		0.43		0.41	0.45	

Table S4.28: Values of population growth rate at 20°C.

Clone / Replicate	CW	EF	EY	RK	IH	NK	GV	RD	ID	FN
1	0.46	0.41	0.35	0.45		0.37	0.40		0.44	0.49
2	0.43	0.41		0.00	0.40	0.39	0.34		0.39	0.34
3	0.45	0.39		0.42		0.39	0.42	0.43	0.44	0.41

Table S4.29: Values of population growth rate at 20°C.

Clone / Replicate	SY	AW	DU	HU	YW	IS	EW	CR	RU	RJ
1	0.38	0.36	0.42	0.41	0.39	0.34		0.42	0.44	0.41
2	0.00		0.40	0.36	0.42	0.00	0.43	0.37	0.40	0.41
3	0.42	0.36	0.39	0.33	0.41	0.31		0.48	0.40	0.44

Table S4.30: Values of population growth rate at 20°C.

Clone / Replicate	BA	OA	GW	KE	PV	JH	JY	EN	VF	CE
1		0.40	0.44	0.43	0.40	0.41		0.32	0.46	0.38
2		0.36	0.42	0.40	0.53	0.38	0.41	0.22	0.45	0.35
3		0.35	0.00	0.43	0.33	0.40	0.43	0.08	0.50	0.37

Table S4.31: Values of population growth rate at 20°C and Cd.

Clone / Replicate	A	B	C	D	F	G	H	J	K	N
1	0.33	0.33	0.33	0.37	0.36	0.34	0.33	0.37	0.33	0.37
2		0.34	0.34	0.36	0.34	0.34	0.34	0.35	0.38	0.35
3	0.38	0.32	0.29	0.35	0.36	0.34	0.35	0.35	0.37	0.37

Table S4.32: Values of population growth rate at 20°C and Cd.

Clone / Replicate	O	P	R	S	U	V	W	Y	E	I
1	0.37	0.37	0.38	0.42	0.40	0.42	0.31	0.42	0.43	
2	0.43	0.37		0.41	0.43	0.41	0.41	0.42	0.40	
3		0.35		0.40		0.43	0.29	0.40	0.43	

Table S4.33: Values of population growth rate at 20°C and Cd.

Clone / Replicate	CW	EF	EY	RK	IH	NK	GV	RD	ID	FN
1	0.45	0.39	0.00			0.33	0.34		0.00	0.35
2	0.41	0.38		0.33	0.33	0.36	0.38		0.00	0.00
3	0.42	0.38		0.38		0.36	0.35	0.41	0.46	0.40

Table S4.34: Values of population growth rate at 20°C and Cd.

Clone / Replicate	SY	AW	DU	HU	YW	IS	EW	CR	RU	RJ
1	0.36	0.34	0.39	0.00	0.43	0.36		0.37	0.42	0.34
2	0.00		0.45	0.35	0.42	0.33		0.37	0.38	0.38
3	0.39		0.40	0.33	0.37	0.12		0.41	0.41	0.36

Table S4.35: Values of population growth rate at 20°C and Cd.

Clone / Replicate	RJ	BA	OA	GW	KE	PV	JH	JY	EN	VF	CE
1	0.34		0.33	0.42	0.45	0.41	0.39		0.25	0.47	0.37
2	0.38		0.34	0.39	0.39	0.52	0.33	0.42	0.25	0.45	0.36
3	0.36		0.36	0.00	0.41	0.32	0.34	0.41	0.15	0.41	0.00

Table S4.36: Values of population growth rate at 20°C and Cd.

Clone / Replicate	FY	FD	SP	HR	SD	NA	RN	DA	NU
1	0.39	0.39	0.40	0.42	0.42	0.30	0.39	0.35	0.42
2			0.50	0.43	0.42	0.34	0.41	0.36	0.43
3		0.26	0.40	0.44	0.44	0.34	0.41	0.33	

Table S4.37: Values of population growth rate at 24°C.

Clone / Replicate	A	B	C	D	F	G	H	J	K	N
1	0.41	0.49	0.49	0.51	0.43	0.44	0.49	0.45	0.49	0.44
2	0.43	0.48	0.48	0.44	0.41	0.49	0.46	0.46	0.41	0.44
3	0.25		0.00	0.44	0.44	0.53	0.49	0.45	0.42	0.44

Table S4.38: Values of population growth rate at 24°C.

Clone / Replicate	O	P	R	S	U	V	W	Y	E	I
1	0.45	0.44	0.40	0.50	0.47	0.43	0.42	0.51	0.42	
2	0.47	0.47	0.38	0.44	0.46	0.42	0.46	0.54	0.42	
3	0.41	0.45	0.41	0.45	0.51	0.44		0.49	0.42	

Table S4.39: Values of population growth rate at 24°C.

Clone / Replicate	CW	EF	EY	RK	IH	NK	GV	RD	ID	FN
1	0.42	0.45		0.31	0.42	0.34	0.39	0.45	0.00	0.44
2	0.41	0.47		0.45	0.41	0.38	0.45	0.00	0.42	0.42
3	0.45	0.38		0.36	0.00	0.28	0.44	0.40	0.00	

Table S4.40: Values of population growth rate at 24°C.

Clone / Replicate	SY	AW	DU	HU	YW	IS	EW	CR	RU	RJ
1	0.43	0.42	0.45	0.44	0.40	0.00	0.48	0.45	0.45	0.42
2	0.00	0.36	0.43	0.39	0.55		0.50	0.00	0.46	0.44
3	0.48	0.43	0.44	0.43	0.47		0.44	0.43	0.48	0.00

Table S4.41: Values of population growth rate at 24°C.

Clone / Replicate	BA	OA	GW	KE	PV	JH	JY	EN	VF	CE
1	0.36	0.14	0.43	0.46	0.42	0.00	0.46	0.00	0.44	0.30
2	0.46	0.00	0.51	0.43	0.47	0.45	0.42	0.09	0.44	0.38
3		0.24		0.45	0.42	0.48	0.52		0.43	0.37

Table S4.42: Values of population growth rate at 24°C.

Clone / Replicate	FY	FD	SP	HR	SD	NA	RN	DA	NU
1	0.52	0.42	0.45	0.51	0.57	0.29	0.48	0.48	0.38
2	0.48	0.45	0.43	0.49	0.54	0.47	0.46	0.49	0.48
3	0.46	0.47		0.48	0.49	0.43	0.50	0.46	0.40

Table S4.43: Values of population growth rate at 24°C and Cd.

Clone / Replicate	A	B	C	D	F	G	H	J	K	N
1	0.19	0.00	0.00	0.40	0.28	0.00	0.17	0.32	0.30	0.31
2	0.13	0.00	0.00	0.34	0.33	0.00	0.18	0.26	0.00	0.20
3	0.21		0.37	0.00	0.33	0.00	0.15	0.25	0.00	0.00

Table S4.44.: Values of population growth rate at 24°C and Cd.

Clone / Replicate	O	P	R	S	U	V	W	Y	E	I
1	0.00	0.20	0.00	0.17	0.28	0.00	0.09	0.35	0.16	
2	0.00	0.20	0.00	0.26	0.26	0.18	0.00	0.00	0.22	
3	0.00	0.16	0.00	0.00	0.23	0.00		0.41	0.20	

Table S4.45.: Values of population growth rate at 24°C and Cd.

Clone / Replicate	CW	EF	EY	RK	IH	NK	GV	RD	ID	FN
1	0.00	0.00		0.00	0.00	0.20	0.19	0.30	0.00	0.39
2	0.10	0.23		0.23	0.00	0.00	0.22	0.31	0.25	0.38
3	0.00	0.00		0.16	0.00	0.16	0.00	0.16	0.00	

Table S4.46: Values of population growth rate at 24°C and Cd.

Clone / Replicate	SY	AW	DU	HU	YW	IS	EW	CR	RU	RJ
1	0.00	0.00	0.28	0.00	0.33	0.00	0.30	0.27	0.32	0.12
2	0.00	0.27	0.32	0.00	0.00		0.24	0.28	0.29	0.24
3	0.10	0.33	0.39	0.18	0.00		0.20	0.17	0.20	0.27

Table S4.47: Values of population growth rate at 24°C and Cd.

Clone / Replicate	BA	OA	GW	KE	PV	JH	JY	EN	VF	CE
1	0.23	0.18	0.06	0.30	0.22	0.38	0.00	0.00	0.25	0.25
2	0.32	0.36	0.00	0.28	0.14	0.28	0.08	0.00	0.32	0.17
3		0.00		0.14	0.29	0.30	0.00		0.26	0.00

Table S4.48: Values of population growth rate at 24°C and Cd.

Clone / Replicate	FY	FD	SP	HR	SD	NA	RN	DA	NU
1	0.30	0.42	0.10	0.32	0.34	0.00	0.35	0.00	0.23
2	0.00	0.32	0.00	0.38	0.27	0.30	0.00	0.00	0.37
3	0.00	0.00		0.26	0.00	0.15	0.00	0.00	0.18

Table S4.49: Asymptotic covariance matrix of estimates of total reproduction at 20°C.

Covariance parameters	additive genetic variance	dominance genetic variance	residual genetic variance
additive genetic variance	36486	-30338	-1210.01
dominance genetic variance		328158	-22477
residual genetic variance			18966

Table S4.50: Asymptotic covariance matrix of estimates of total reproduction at 20°C and Cd.

Covariance parameters	additive genetic variance	dominance genetic variance	residual genetic variance
additive genetic variance	28896	-23348	-1091.66
dominance genetic variance		166955	-29044
residual genetic variance			20595

Table S4.51: Asymptotic covariance matrix of estimates of total reproduction at 24°C.

Covariance parameters	additive genetic variance	dominance genetic variance	residual genetic variance
additive genetic variance	79451	-27317	-13522
dominance genetic variance		353691	-58597
residual genetic variance			69360

Table S4.52: Asymptotic covariance matrix of estimates of total reproduction at 24°C and Cd.

Covariance parameters	additive genetic variance	dominance genetic variance	residual genetic variance
additive genetic variance	2589.7921	-2471.69	80.5209
dominance genetic variance		11722	-1485.30
residual genetic variance			908.21

Table S4.53: Asymptotic covariance matrix of estimates of population growth rate at 24°C.

Covariance parameters	additive genetic variance	dominance genetic variance	residual genetic variance
additive genetic variance	1.4E-05	-2.84E-06	-1.86E-06
dominance genetic variance		4.8E-05	-9.77E-06
residual genetic variance			5.00E-06

Table S4.54: Asymptotic covariance matrix of estimates of population growth rate at 24°C and Cd.

Covariance parameters	additive genetic variance	dominance genetic variance	residual genetic variance
additive genetic variance	5.51E-06	-4.53E-06	-2.18E-07
dominance genetic variance		3.70E-05	-4.41E-06
residual genetic variance			3.65E-06

S4.1.: The Animal model

Theory

Assuming a single fixed factor (the population mean μ), and assuming a single observation for each individual *i* of a total of *k* individuals, the observation y_i for individual *i* is expressed as:

$$y_i = \mu + a_i + d_i + e_i \qquad \text{(S4.1)}$$

Where y_i is the observed trait value for individual i

a_i is the additive genetic value of individual i (random effect) (breeding value)

d_i is the dominance genetic value of individual i (random effect)

e_i is a residual deviation

The model can be expressed in matrix form (linear mixed model):

$$Y = X\beta + Z_1 u_1 + Z_2 u_2 + e \qquad \text{(S4.2)}$$

Where

$$Y = \begin{pmatrix} y_1 \\ y_2 \\ \dots \\ y_k \end{pmatrix}, X = \begin{pmatrix} 1 \\ 1 \\ \dots \\ 1 \end{pmatrix} =, \beta = \mu, Z_1 = Z_2 = \begin{pmatrix} 1 & 0 & \dots & 0 \\ 0 & 1 & \dots & 0 \\ \dots & \dots & \dots & \dots \\ 0 & 0 & \dots & 1 \end{pmatrix}, u_1 = \begin{pmatrix} a_1 \\ a_2 \\ \dots \\ a_k \end{pmatrix}, u_2 = \begin{pmatrix} d_1 \\ d_2 \\ \dots \\ d_k \end{pmatrix}, e = \begin{pmatrix} e_1 \\ e_2 \\ \dots \\ e_k \end{pmatrix} \qquad \text{(S4.3)}$$

Assume that u_1, u_2, and e are uncorrelated and their distributions have means equal to 0. Further, denote the (k x k) covariance matrix for the vector e of residual errors by R and the (k x k) covariance matrix for the vectors u_1 (random additive genetic effects) and u_2 (random dominance genetic effects) by G_1 and G_2, respectively:

$u_1 \sim (0, G_1)$, $u_2 \sim (0, G_2)$, $e \sim (0, R)$ (S4.4)

Substituting (S4.3) in (S4.2), we get:

$$Y \sim (X\mu, V) \qquad \text{(S4.5)}$$

With the covariance matrix for the vector of observations Y equal to

$$V = Z_1 G_1 Z_1^T + Z_2 G_2 Z_2^T + R \qquad \text{(S4.6)}$$

Given the single observation per individual and thus the structure of the Z matrixes (ones on the diagonal, zeroes off-diagonal), this simplifies to:

$$V = G_1 + G_2 + R \qquad \text{(S4.7)}$$

We will usually assume that residual errors have constant variance and are uncorrelated, so that R is a diagonal matrix, with:

$$R = \sigma_e^2 I \qquad \text{(S4.8)}$$

The matrix G_1 describes the covariances among the random additive genetic effects and follows from standard results for the covariances between relatives. The additive genetic covariance between two relatives i and j is given by $2\Theta_{ij}\sigma_a^2$, i.e. by twice the coefficient of coancestry times the additive genetic variance in the population. Hence,

$$G_1 = \sigma_a^2 A \qquad \text{(S4.9)}$$

Where the additive genetic relationship matrix A has elements

$$A_{ij} = 2\Theta_{ij} \qquad \text{(S4.10)}$$

Coefficients of coancestry for different relationships are given in Table S4.55.

Similarly, the matrix G_2 describes the covariances among the random dominance genetic effects and also follows from standard results for the covariances between relatives. The dominance genetic covariance between two relatives i and j is given by $\Delta_{ij}\sigma_d^2$, i.e. by the coefficient of fraternity times the dominance genetic variance in the population. Hence,

$$G_2 = \sigma_d^2 D \qquad \text{(S4.11)}$$

Where the dominance genetic relationship matrix D has elements

$$D_{ij} = \Delta_{ij} \qquad \text{(S4.12)}$$

Substituting (8), (10) and (12) in (5), we get:

$$Y \sim \left(X\mu, \sigma_a^2 A + \sigma_d^2 D + \sigma_e^2 I \right) \qquad \text{(S4.13)}$$

The parameters $\mu, \sigma_a^2, \sigma_d^2, \sigma_e^2$ of this linear model (and their 95% confidence limits) can be fitted to the data with restricted maximum likelihood estimation (REML). From these estimates we can further calculate heritability (the sum in the denominator is the total phenotypic variance):

broad-sense heritability: $H^2 = \frac{\sigma_a^2 + \sigma_d^2}{\sigma_a^2 + \sigma_d^2 + \sigma_e^2}$

narrow-sense heritability: $H^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_d^2 + \sigma_e^2}$

Based on these calculations, we can determine if a trait is genetically heritable or not.

Table S4.55: Coeficients of coancestry (θ_{ij}) and coefficients of fraternity (θ_{ij})

Relationship	θ_{ij}	θ_{ij}
Clone-mates (e.g. monozygotic twins)	1/2	1
Parent-offspring	1/4	0
Full-Sib	1/4	1/4
Half-Sib	1/8	0

S4.2:Estimation of the different parameters (h^2, H^2, CV_A, CV_G,) and their variances

These are based on Lynch and Walsch (1998).

Based Lynch and Walsch (1998) equation A1.4.b pp 809 and equation A1.7c pp 811:

$$E(f) = f + \sigma_x^2 \frac{\partial^2 f}{2\,\partial x^2} + \sigma^2{}_y \frac{\partial^2 f}{2\,\partial y^2} + \sigma_z^2 \frac{\partial^2 f}{2\,\partial z^2} + \sigma_{xy} \frac{\partial^2 f}{\partial x\,\partial y} + \sigma_{xz} \frac{\partial^2 f}{\partial x\,\partial z} + \sigma_{yz} \frac{\partial^2 f}{\partial y\,\partial z}$$

$$\sigma^2(f) = \sigma_x^2 \left(\frac{\partial f}{\partial x}\right)^2 + \sigma^2{}_y \left(\frac{\partial f}{\partial y}\right)^2 + \sigma_z^2 \left(\frac{\partial f}{\partial z}\right)^2 + 2\sigma_{xy} \frac{\partial f}{\partial x}\frac{\partial f}{\partial y} + 2\sigma_{xz} \frac{\partial f}{\partial z}\frac{\partial f}{\partial x} + 2\sigma_{yz} \frac{\partial f}{\partial y}\frac{\partial f}{\partial z}$$

where x= additive genetic variance

y= dominance genetic variance

z= environmental genetic variance

S4.2.1.Estimation of Broad sense heritability (H^2) and it's variance of a trait

$$H^2 = \frac{V_a + V_d}{V_a + V_d + V_e} = \mathrm{f} = \frac{x + y}{x + y + z}$$

$$\frac{\partial f}{\partial x} = \frac{\partial f}{\partial y} = \frac{z}{(x + y + z)^2}$$

$$\frac{\partial^2 f}{\partial x^2} = \frac{\partial^2 f}{\partial y^2} = \frac{-2z}{(x + y + z)^3}$$

$$\frac{\partial f}{\partial z} = \frac{-(x + y)}{(x + y + z)^2}$$

$$\frac{\partial^2 f}{\partial z^2} = \frac{2(x + y)}{(x + y + z)^3}$$

$$\frac{\partial^2 f}{\partial x \partial} = \frac{-2z}{(x + y + z)^3}$$

$$\frac{\partial^2 f}{\partial y \partial} = \frac{\partial^2 f}{\partial x \partial} = \frac{x + y - z}{(x + y + z)^3}$$

S4.2.2.Estimation of narrow sense heritability (h^2) and it's variance of a trait

$$\mathrm{h}^2 = \frac{\mathrm{Va}}{\mathrm{Va+Vd+Ve}} = f = \frac{\mathrm{x}}{\mathrm{x+y+z}}$$

$$\frac{\partial \mathrm{f}}{\partial \mathrm{x}} = \frac{\mathrm{y} + \mathrm{z}}{?\mathrm{x} + \mathrm{y} + \mathrm{z}?^2}$$

$$\frac{\partial^2 \mathrm{f}}{\partial \mathrm{x}^2} = \frac{-2?\mathrm{y} + \mathrm{z}?}{?\mathrm{x} + \mathrm{y} + \mathrm{z}?^3}$$

$$\frac{\partial \mathrm{f}}{\partial \mathrm{y}} = \frac{\partial \mathrm{f}}{\partial \mathrm{z}} = \frac{-\mathrm{x}}{?\mathrm{x} + \mathrm{y} + \mathrm{z}?^2}$$

$$\frac{\partial^2 \mathrm{f}}{\partial \mathrm{y}^2} = \frac{\partial^2 \mathrm{f}}{\partial \mathrm{z}^2} = \frac{2\mathrm{x}}{(\mathrm{x} + \mathrm{y} + \mathrm{z})^3}$$

$$\frac{\partial^2 \mathrm{f}}{\partial \mathrm{x}\, \partial \mathrm{y}} = \frac{\partial^2 \mathrm{f}}{\partial \mathrm{x}\, \partial \mathrm{z}} = -\frac{(-\mathrm{x} + \mathrm{y} + \mathrm{z})}{(\mathrm{x} + \mathrm{y} + \mathrm{z})^3}$$

$$\frac{\partial^2 \mathrm{f}}{\partial \mathrm{y}\, \partial \mathrm{z}} = \frac{2\mathrm{x}}{(\mathrm{x} + \mathrm{y} + \mathrm{z})^3}$$

S4.2.3:Estimation of genetic coefficient of variation (CV_G)

$$CV_G = 100 * f$$

μ= population mean

$$f = \frac{? \overline{x + y}}{\mu}$$

$$E(f) = \frac{\sqrt{x+y}}{\mu} \frac{1}{2} x\left(\frac{d^2 f}{dx^2} + \frac{1}{2} y \frac{d^2 f}{dy^2} + \sigma_{xy} \frac{d^2 f}{dx}\right)$$

$$\sigma_f^2 = x \left(\frac{df}{dx}\right)^2 + + y \left(\frac{df}{dy}\right)^2 + \mu\left(\frac{df}{d\mu}\right)^2 + + 2\sigma_{xy} \frac{df}{dxd} \quad y$$

S4.2.4.:Estimation of coefficient of additive genetic variation of a trait (CV_A)

$$CV_G = 100 * f$$

$$f = \sqrt{x} / \mu$$

Based on Lynch and Walsch (1998):

$$E?f? = \frac{\sqrt{x}}{\mu} + \ x \frac{d^2 f}{dx^2} + \frac{1}{2} \mu \frac{d^2 f}{d\mu^2}$$

$$\sigma_f^2 = x\left(\frac{df}{dx}\right)^2 + \mu\left(\frac{df}{d\mu}\right)^2$$

Table S5.1: Physico-chemical measurements during tests. Values represent mean values ± standard deviation.

Nominal Cd concentration (µg Cd/L)		DOC (mg C/L)	pH	Cd concentration (µg Cd/L)	Mean Cd concentration (µg Cd/L)
0	NM	3.98 ± 0.13	7.62	0.21 ± 0.12	0.19 ± 0.09
	OM	7.17 ± 0.81	7.65 ± 0.32	0.16 ± 0.08	
5	NM	4.05 ± 0.23	7.63	4.57 ± 0.27	4.37 ± 0.37
	OM	7.60 ± 0.68	7.82 ± 0.35	4.16 ± 0.37	

Table S5.2: Values of total reproduction of KNO52 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	18	0	76	11	0	102	44	70	122	131	144
2	34	41	66	120	13	118	29	63	106	120	136
3		49	114		57	46	51	74	115	126	44

Table S5.3: Values of total reproduction of KNO52 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1		74	91		28	79	58	63	28	21	27
2	93	75	74	87	25	70	41	62	32	104	117
3		20	97		2	58	34	36	73	84	110

Table S5.4: Values of population growth rate of KNO52 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.29	0.00	0.33	0.24	0.00	0.40	0.37	0.36	0.36	0.45	0.40
2	0.31	0.35	0.35	0.43	0.27	0.36	0.36	0.38	0.36	0.39	0.40
3		0.42	0.44		0.29	0.38	0.40	0.40	0.35	0.40	0.40

Table S5.5: Values of population growth rate of KNO52 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1		0.37	0.39		0.19	0.32	0.31	0.34	0.21	0.30	0.20
2	0.36	0.39	0.38	0.40	0.22	0.29	0.31	0.28	0.24	0.37	0.37
3		0.28	0.43		0.06	0.32	0.32	0.35	0.34	0.36	0.36

Table S5.6: Values of reproduction at first brood of KNO52 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	5		14	11		14	10	11	11	16	14
2	20	17	9	28	5	11	11	14	15	13	13
3		10	14		7	12	9	11	11	15	17

Table S5.7: Values of reproduction at first brood of KNO52 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1		10	13		6	8	4	9	8	9	7
2	10	10	12	8	6	3	6	8	5	17	9
3		11	13		2	6	4	8	12	7	8

Table S5.8: Values of maturation rate of KNO52 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.13		0.10	0.10		0.13	0.13	0.13	0.11	0.14	0.13
2	0.09	0.11	0.13	0.14	0.13	0.11	0.13	0.13	0.11	0.13	0.13
3		0.14	0.14		0.10	0.13	0.14	0.14	0.11	0.13	0.13

Table S5.9: Values of maturation rate of KNO52 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1		0.11	0.13		0.07	0.11	0.10	0.10	0.08	0.09	0.07
2	0.13	0.13	0.13	0.13	0.09	0.11	0.10	0.09	0.09	0.09	0.11
3		0.10	0.13		0.09	0.13	0.10	0.13	0.11	0.11	0.11

Table S5.10: Values of total reproduction of ZW4 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	114	103	99	130	80	83	94	0	42	38	70
2	83	110	82	113	0	78	85	126	71	0	78
3	109	75	106	123	0	80	85	108	0	45	87

Table S5.11: Values of total reproduction during 21 days of ZW4 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	63	82	73	88	74	0	0	92	60	67	82
2	58	70	71	71	100	0	0	75	87	0	68
3	77	59	93	100	0	0	7	76		31	68

Table S5.12: Values of population growth rate of ZW4 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.39	0.37	0.37	0.43	0.43	0.36	0.38	0.00	0.41	0.31	0.36
2	0.39	0.33	0.40	0.36	0.00	0.41	0.36	0.39	0.39	0.00	0.37
3	0.41	0.35	0.39	0.44	0.00	0.43	0.37	0.39	0.00	0.35	0.38

Table S5.13: Values of population growth rate of ZW4 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.30	0.33	0.35	0.36	0.36	0.00	0.00	0.32	0.34	0.30	0.35
2	0.32	0.31	0.32	0.38	0.36	0.00	0.00	0.33	0.31	0.00	0.31
3	0.34	0.31	0.36	0.39	0.00	0.00	0.22	0.33		0.23	0.30

Table S5.14: Values of reproduction at first brood of ZW4 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	25	15	9	17	15	2	11		14	6	7
2	15	12	11	25		9	7	10	11		9
3	14	18	14	20		14	8	12		10	12

Table S5.15: Values of reproduction at first brood of ZW4 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	8	14	10	11	8			6	6	16	5
2	15	8	8	12	16			7	11		6
3	12	10	12	14			5	8		1	

Table S5.16: Values of maturation rate of ZW4 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.11	0.11	0.13	0.13	0.14	0.13	0.13		0.13	0.13	0.13
2	0.13	0.10	0.14	0.10		0.14	0.13	0.13	0.13		0.13
3	0.13	0.10	0.13	0.13		0.14	0.13	0.13		0.13	0.13

Table S5.17: Values of maturation rate of ZW4 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.10	0.10	0.13	0.13	0.13			0.11	0.13	0.09	0.13
2	0.10	0.10	0.11	0.13	0.11			0.11	0.10		0.13
3	0.11	0.10	0.11	0.13			0.13	0.11		0.09	0.11

Table S5.18: Values of total reproduction of TER2 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10
1	0	153	114	83	118	98	121	138	116	0
2	0	39	108	80	107	97	26	108	105	0
3	91	168	0	85	97	89	116	0	107	68

Table S5.19: Values of total reproduction of TER2 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10
1	39	16	0	0	3	74	65	115	70	0
2	3	23	0	0	0	41	4	91	104	0
3	12	21	0	0	0	60	17	68	65	85

Table S5.20: Values of population growth rate of TER2 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10
1	0.00	0.39	0.37	0.34	0.36	0.35	0.39	0.39	0.34	0.00
2	0.00	0.39	0.33	0.33	0.36	0.34	0.36	0.35	0.38	0.00
3	0.44	0.41	0.00	0.35	0.33	0.38	0.39	0.00	0.44	0.35

Table S5.21: Values of population growth rate of TER2 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10
1	0.30	0.27	0.00	0.00	0.11	0.36	0.27	0.37	0.30	0.00
2	0.14	0.28	0.00	0.00	0.00	0.35	0.17	0.36	0.37	0.00
3	0.28	0.29	0.00	0.00	0.00	0.35	0.00	0.28	0.30	0.36

Table S5.22: Values of reproduction at first brood of TER2 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10
1		14	11	8	13	7	15	19	22	
2		18	3	8	14	8	14	2	18	
3	14	16		9	15	13	15		14	10

Table S5.23: Values of reproduction at first brood of TER2 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10
1	14	8			3	7	5	14	13	
2	3	5				6	4	13	11	
3	7	8				8		12	7	14

Table S5.24: Values of maturation rate of TER2 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10
1		0.13	0.13	0.11	0.11	0.13	0.13	0.11	0.09	
2		0.13	0.13	0.11	0.11	0.13	0.13	0.11	0.11	
3	0.14	0.13		0.13	0.09	0.13	0.13		0.14	0.11

Table S5.25: Values of maturation rate of TER2 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10
1	0.11	0.11			0.10	0.14	0.11	0.11	0.09	
2	0.13	0.11				0.13	0.13	0.11	0.13	
3	0.13	0.11				0.13		0.09	0.11	0.11

Table S5.26: Values of total reproduction of TER1 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	14	80	0	148	56	94	122	107	133	166	117	119
2	30	72	0	137	77	75	104	102	126	159	31	103
3	106	29	0	114	73	0	14	99	119	121	44	114

Table S5.27: Values of total reproduction of TER1 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	11	97	120	2	48	32	94	88	87	117	94	88
2	0	102	5	100	32	40	80	90	75	113	104	98
3	5	27		94	63	0	91	89	81	141	116	107

Table S5.28: Values of population growth rate of TER1 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.17	0.39	0.00	0.40	0.31	0.40	0.39	0.37	0.38	0.35	0.38	0.42
2	0.30	0.38	0.00	0.40	0.32	0.33	0.39	0.36	0.38	0.37	0.38	0.39
3	0.36	0.35	0.00	0.38	0.33	0.00	0.33	0.38	0.41	0.36	0.39	0.40

Table S5.29: Values of population growth rate of TER1 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.17	0.39	0.38	0.08	0.28	0.30	0.36	0.36	0.42	0.35	0.36	0.40
2	0.00	0.37	0.20	0.36	0.29	0.23	0.37	0.35	0.41	0.36	0.37	0.35
3	0.10	0.33		0.36	0.31	0.00	0.38	0.35	0.42	0.34	0.38	0.41

Table S5.30: Values of reproduction at first brood of TER1 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	2	11		18	11	14	23	16	18	18	17	17
2	12	10		25	5	17	13	14	9	12	11	11
3	13	10		11	11		14	20	8	15	17	12

Table S5.31: Values of reproduction at first brood of TER1 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	3	10	17	2	6	10	15	16	27	10	9	13
2		9	5	8	4	1	9	12	25	14	9	15
3	5	12		7	8		12	15	9	16	13	15

Table S5.32: Values of maturation rate of TER1 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.08	0.13		0.11	0.10	0.13	0.11	0.11	0.11	0.10	0.11	0.13
2	0.10	0.13		0.11	0.11	0.10	0.13	0.11	0.13	0.11	0.13	0.13
3	0.11	0.13		0.13	0.11		0.13	0.11	0.14	0.11	0.13	0.13

Table S5.33: Values of maturation rate of TER1 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.11	0.13	0.11	0.11	0.10	0.13	0.11	0.11	0.13	0.11	0.13	0.13
2		0.13	0.13	0.13	0.11	0.08	0.13	0.11	0.13	0.11	0.13	0.11
3	0.06	0.11		0.13	0.11		0.13	0.11	0.14	0.10	0.13	0.13

Table S5.34: Values of total reproduction of MO population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	114	107	82	87		100	93	116	0	62	78	
2	109	107	110	87		119	107	132	112	69	97	116
3	109	104	84	83		101	138	105	122	67	87	

Table S5.35: Values of total reproduction of MO population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	84	65	28		61	112	96	77		65	80	
2	83		52	60		52	78	82	105		78	
3	75	82	72	31		95	93	78	98	54	69	

Table S5.36: Values of population growth rate of MO population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.30	0.38	0.31	0.36		0.34	0.35	0.38	0.00	0.29	0.31	
2	0.35	0.34	0.38	0.35		0.37	0.36	0.37	0.37	0.30	0.31	0.35
3	0.38	0.36	0.35	0.34		0.37	0.38	0.33	0.38	0.33	0.31	

Table S5.37: Values of population growth rate of MO population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.31	0.29	0.30		0.33	0.34	0.36	0.30		0.28	0.32	
2	0.30		0.27	0.36		0.31	0.32	0.34	0.33		0.29	
3	0.34	0.30	0.30	0.27		0.37	0.37	0.35	0.37	0.32	0.28	

Table S5.38: Values of reproduction at first brood of MO population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	14	19	8	18		16	9	13		7	7	
2	17	17	12	12		14	9	17	12	7	6	19
3	18	17	13	10		16	18	17	11	11	8	

Table S5.39: Values of reproduction at first brood of MO population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	11	9	9		17	7	8	2		8	8	
2	9		13	14		11	7	7	8		5	
3	13	10	12	7		16	10	8	11	10	6	

Table S5.40: Values of maturation rate of MO population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.09	0.11	0.10	0.11		0.10	0.11	0.13		0.11	0.10	
2	0.10	0.10	0.13	0.11		0.11	0.13	0.11	0.13	0.10	0.10	0.11
3	0.11	0.11	0.11	0.11		0.11	0.11	0.10	0.13	0.11	0.10	

Table S5.41: Values of maturation rate of MO population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.10	0.10	0.10		0.10	0.11	0.13	0.11		0.09	0.11	
2	0.10		0.09	0.11		0.10	0.11	0.13	0.11		0.10	
3	0.11	0.10	0.09	0.10		0.11	0.13	0.13	0.13	0.11	0.10	

Table S5.42: Values of total reproduction of OM3 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	73	75	90	98	111	30	89	168	11	121	45
2	85	32	78	0	130	116	133	134	87	104	51
3	80	65	121	111		99	140	130	129	108	63

Table S5.43: Values of total reproduction of OM3 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	72	0	59	67	93	70	64	22	41	119	1
2	71	72	70	61	88	84	94	7	7	123	0
3	66	81	95	67		99	70	13	5	128	0

Table S5.44: Values of population growth rate of OM3 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.33	0.33	0.38	0.39	0.36	0.32	0.33	0.47	0.30	0.41	0.38
2	0.33	0.34	0.34	0.00	0.40	0.40	0.35	0.43	0.37	0.40	0.40
3	0.34	0.39	0.37	0.40		0.39	0.33	0.40	0.40	0.41	0.41

Table S5.45: Values of population growth rate of OM3 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.32	0.00	0.26	0.34	0.32	0.33	0.29	0.17	0.33	0.41	0.00
2	0.33	0.34	0.32	0.32	0.32	0.36	0.28	0.24	0.24	0.36	0.00
3	0.33	0.34	0.31	0.31		0.38	0.27	0.31	0.20	0.37	0.00

Table S5.46: Values of reproduction at first brood of OM3 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	5	13	9	14	14	15	6	26	11	17	14
2	3	9	20		13	14	5	12	9	16	10
3	6	15	3	17		15	10	14	13	15	10

Table S5.47: Values of reproduction at first brood of OM3 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	6		2	9	7	12	2	22	9	13	1
2	4	9	6	5	8	10	6	7	7	10	
3	4	2	7	7		11	6	8	5	11	

Table S5.48: Values of maturation rate of OM3 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.13	0.11	0.13	0.13	0.11	0.11	0.11	0.11	0.13	0.13	0.13
2	0.11	0.11	0.10		0.13	0.13	0.11	0.14	0.13	0.13	0.14
3	0.13	0.13	0.13	0.13		0.13	0.10	0.13	0.13	0.13	0.14

Table S5.49: Values of maturation rate of OM3 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.11		0.13	0.13	0.11	0.11	0.11	0.06	0.13	0.13	0.08
2	0.13	0.11	0.13	0.13	0.11	0.13	0.09	0.13	0.13	0.11	
3	0.13	0.13	0.09	0.11		0.13	0.09	0.14	0.13	0.11	

Table S5.50: Values of total reproduction of OHZ population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	129	135	0	125	0	123	83	122	125	101	129	135
2	0	115	100	0	150	84		100	128	103	0	115
3	143	107	0	132	89	122		133	131	92	143	107

Table S5.51: Values of total reproduction of OHZ population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	67	8	0		28	39	82	92	93	0	67	8
2	59	9	78	0	16	54		82	110	43	59	9
3	96	6	0		19	48		137	76	0	96	6

Table S5.52: Values of population growth rate of OHZ population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.37	0.35	0.00	0.40	0.00	0.38	0.38	0.40	0.38	0.39	0.37	0.35
2	0.00	0.37	0.36	0.00	0.39	0.33		0.34	0.36	0.37	0.00	0.37
3	0.42	0.37	0.00	0.39	0.36	0.40		0.41	0.39	0.37	0.42	0.37

Table S5.53: Values of population growth rate of OHZ population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.31	0.12	0.00		0.33	0.32	0.31	0.36	0.34	0.00	0.31	0.12
2	0.29	0.24	0.33	0.00	0.33	0.34		0.33	0.35	0.29	0.29	0.24
3	0.31	0.20	0.00		0.25	0.31		0.36	0.32	0.00	0.31	0.20

Table S5.54: Values of reproduction at first brood of OHZ population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	16	20		16		11	21	11	12	13	16	20
2		27	13		17	13		17	8	11		27
3	16	25		12	23	21		16	20	18	16	25

Table S5.55: Values of reproduction at first brood of OHZ population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	8	8			9	6	14	11	8		8	8
2	5	9	10		6	13		6	12	7	5	9
3	16	6			2	15		12	17		16	6

Table S5.56: Values of maturation rate of OHZ population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.11	0.10		0.13		0.13	0.11	0.13	0.11	0.13	0.11	0.10
2		0.10	0.11		0.11	0.10		0.10	0.11	0.13		0.10
3	0.13	0.10		0.13	0.10	0.11		0.13	0.11	0.11	0.13	0.10

Table S5.57: Values of maturation rate of OHZ population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.11	0.06			0.13	0.13	0.10	0.13	0.13		0.11	0.06
2	0.11	0.11	0.11		0.06	0.11		0.13	0.11	0.11	0.11	0.11
3	0.09	0.11			0.13	0.11		0.11	0.10		0.09	0.11

Table S5.58: Values of total reproduction during 21 days of KNO17 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	85	120	105	105	109	48	74	124	89	70	161
2	95	143	91	91	120	109	118	133	0	78	139
3	0	109	109	109	97	82	104	135	111	101	130

Table S5.59: Values of total reproduction of KNO17 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	47	44	33	19	0	8	94	96	10	0	139
2	26	64	0	11	0	9	87	12	0	7	106
3	25	71	38	37	3	11	34	103	6	6	105

Table S5.60: Values of population growth rate of KNO17 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.29	0.37	0.39	0.43	0.43	0.39	0.40	0.41	0.34	0.34	0.42
2	0.29	0.40	0.37	0.42	0.40	0.39	0.40	0.40	0.00	0.36	0.41
3	0.00	0.32	0.40	0.41	0.32	0.36	0.38	0.41	0.40	0.39	0.40

Table S5.61: Values of population growth rate of KNO17 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.32	0.33	0.33	0.29	0.00	0.26	0.36	0.40	0.26	0.00	0.39
2	0.24	0.37	0.00	0.30	0.00	0.24	0.35	0.31	0.00	0.19	0.38
3	0.28	0.35	0.35	0.39	0.11	0.26	0.33	0.38	0.20	0.16	0.37

Table S5.62: Values of reproduction at first brood of KNO17 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	12	14	15	18	11	14	15	18	1	10	18
2	11	15	13	12	13	20	16	15		19	16
3		17	17	17	22	19	10	9	13	20	12

Table S5.63: Values of reproduction at first brood of KNO17 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	13	6	10	19		8	9	15	10		11
2	12	11		11		9	12	12		7	14
3	12	15	12	15	3	9	13	12	6	6	11

Table S5.64: Values of maturation rate of KNO17 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.08	0.11	0.13	0.13	0.14	0.13	0.13	0.13	0.13	0.11	0.13
2	0.09	0.13	0.13	0.13	0.13	0.11	0.13	0.13		0.11	0.13
3		0.09	0.13	0.13	0.09	0.11	0.13	0.13	0.13	0.11	0.13

Table S5.65: Values of maturation rate of KNO17 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11
1	0.11	0.13	0.13	0.10		0.13	0.13	0.13	0.11		0.13
2	0.08	0.13		0.13		0.11	0.11	0.13		0.10	0.13
3	0.10	0.11	0.13	0.13	0.10	0.11	0.11	0.13	0.11	0.09	0.13

Table S5.66: Values of total reproduction of OM2 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	114	0	119	146	111	129	145	149	54	132	125	146
2	76	0	123	113	134	13	79	155	57	77	111	108
3	105	66	0	147	121	106	175	117	68	80	111	142

Table S5.67: Values of total reproduction of OM2 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	95	0	22	0	122	14	116	80	10	39	120	13
2	93	0	50	165	22	13	17	89	16	9	117	41
3	90	52		98	117	32	31	39	17	83	67	65

Table S5.68: Values of population growth rate of OM2 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.38	0.00	0.39	0.42	0.40	0.38	0.43	0.42	0.33	0.37	0.50	0.38
2	0.35	0.00	0.38	0.45	0.39	0.28	0.38	0.44	0.39	0.40	0.49	0.38
3	0.40	0.26	0.00	0.45	0.40	0.38	0.45	0.39	0.36	0.40	0.34	0.40

Table S5.69: Values of population growth rate of OM2 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.35	0.00	0.30	0.00	0.36	0.23	0.41	0.42	0.26	0.26	0.46	0.28
2	0.37	0.00	0.34	0.42	0.34	0.28	0.23	0.42	0.31	0.22	0.48	0.32
3	0.35	0.23		0.30	0.40	0.28	0.39	0.31	0.31	0.32	0.34	0.32

Table S5.70: Values of reproduction at first brood of OM2 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	19		14	19	13	13	16	15	18	19	12	19
2	21		20	15	4	13	11	18	22	25	13	18
3	16	9		15	14	14	17	22	1	26	18	13

Table S5.71: Values of reproduction of first brood of OM2 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	10		6		13	12	16	16	10	9	10	13
2	15		13	17	7	13	6	15	16	9	12	16
3	10	4		11	15	12	15	16	14	11	8	10

Table S5.72: Values of maturation rate of OM2 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.11		0.11	0.13	0.13	0.11	0.13	0.13	0.10	0.11	0.17	0.11
2	0.10		0.11	0.14	0.14	0.11	0.13	0.13	0.11	0.11	0.17	0.11
3	0.13	0.09		0.14	0.13	0.13	0.13	0.11	0.13	0.11	0.10	0.13

Table S5.73: Values of maturation rate of OM2 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.11		0.11		0.11	0.09	0.13	0.13	0.11	0.11	0.17	0.11
2	0.11		0.11	0.13	0.13	0.11	0.13	0.13	0.11	0.10	0.17	0.11
3	0.11	0.07		0.09	0.13	0.11	0.13	0.11	0.11	0.11	0.13	0.13

Table S5.74: Values of total reproduction of LRV population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	95	77	130	156	123	162	130	66	105	148	157	169
2	110	43	120	128	0	112	126	144	114	142	0	175
3	104	102	113	127	0	141	105		118	111	140	144

Table S5.75: Values of total reproduction of LRV population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	93	57	111	16	92	118	32	0	121	107	88	128
2	91	63	151	97	0	135	8	3	124	55	59	168
3	0	21	47	79	0	99	52		109	120	0	140

Table S5.76: Values of population growth rate in LRV population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.35	0.38	0.39	0.42	0.42	0.40	0.38	0.41	0.39	0.44	0.43	0.44
2	0.36	0.33	0.40	0.43	0.00	0.38	0.39	0.42	0.41	0.46	0.00	0.46
3	0.40	0.40	0.39	0.40	0.00	0.40	0.39		0.42	0.43	0.44	0.44

Table S5.77: Values of population growth rate in LRV population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.34	0.31	0.38	0.31	0.38	0.39	0.35	0.00	0.43	0.39	0.34	0.39
2	0.33	0.30	0.38	0.36	0.00	0.38	0.22	0.07	0.39	0.42	0.24	0.45
3	0.00	0.35	0.40	0.36	0.00	0.36	0.33		0.39	0.40	0.00	0.41

Table S5.78: Values of reproduction at first brood of LRV population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	11	11	8	17	18	16	20	15	13	17	19	13
2	13	1	14	20		18	10	14	10	16		16
3	15	13	26	22		13	8		12	19	17	19

Table S5.79: Values of reproduction at first brood of LRV population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	11	10	10	16	13	12	11		10	10	14	14
2	8	7	9	12		19	5	3	18	12	28	15
3		13	22	15		11	16		16	11		15

Table S5.80: Values of maturation rate of LRV population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.11	0.13	0.13	0.13	0.13	0.11	0.11	0.13	0.13	0.13	0.13	0.14
2	0.11	0.13	0.13	0.13		0.11	0.13	0.13	0.14	0.14		0.14
3	0.13	0.13	0.11	0.11		0.13	0.13		0.14	0.13	0.13	0.13

Table S5.81: Values of maturation rate of LRV population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.11	0.10	0.13	0.11	0.13	0.13	0.13		0.14	0.13	0.11	0.11
2	0.11	0.10	0.13	0.11		0.11	0.11	0.07	0.13	0.14	0.06	0.14
3		0.13	0.13	0.11		0.11	0.11		0.13	0.13		0.13

Table S5.82: Values of total reproduction during 21 days of KNO15 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	119	114	142	175	173	157	100	111	81	90	87	27
2	167	72	149	177	162	145	57			136	106	145
3		126	97	64	165	46	109				88	131

Table S5.83: Values of total reproduction during 21 days of KNO15 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	78	10	87	92	98	40	90	140	61	60	65	23
2	106	7	22	111	81	52	73			13	71	72
3		24	41	29	42	30					65	95

Table S5.84: Values of population growth rate of KNO15 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.40	0.38	0.43	0.40	0.41	0.42	0.38	0.37	0.32	0.37	0.35	0.35
2	0.41	0.39	0.43	0.41	0.40	0.39	0.43			0.40	0.39	0.40
3		0.45	0.40	0.40	0.41	0.38	0.40				0.38	0.40

Table S5.85: Values of population growth rate of KNO15 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.32	0.29	0.35	0.35	0.36	0.31	0.39	0.39	0.27	0.31	0.33	0.33
2	0.36	0.24	0.26	0.35	0.37	0.31	0.41			0.29	0.34	0.37
3		0.37	0.28	0.32	0.28	0.34	0.00				0.30	0.34

Table S5.86: Values of reproduction at first brood of KNO15 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	13	8	11	14	16	29	11	22	25	16	16	10
2	21	9	14	15	12	13	12			14	21	13
3		12	11	16	23	18	14				11	15

Table S5.87: Values of reproduction at first brood of KNO15 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	12	10	7	10	9	11	14	9	10	12	12	8
2	8	7	10	9	9	5	12			13	13	12
3		8	10	12	11	12					6	10

Table S5.88: Values of maturation rate of KNO15 population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.11	0.13	0.14	0.11	0.11	0.11	0.13	0.10	0.09	0.11	0.11	0.13
2	0.11	0.13	0.13	0.11	0.11	0.11	0.14			0.13	0.11	0.13
3		0.14	0.13	0.11	0.11	0.11	0.13				0.13	0.13

Table S5.89: Values of maturation rate of KNO15 population in Cd treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.11	0.13	0.13	0.11	0.11	0.11	0.13	0.13	0.09	0.11	0.11	0.13
2	0.11	0.13	0.11	0.11	0.11	0.11	0.14			0.11	0.11	0.13
3		0.14	0.11	0.11	0.11	0.11					0.11	0.11

Table S5.90: Physico-chemical measurements in the water samples of the different ponds. Values represent mean values ± standard deviation.

Pond	Ni (µg/L)	Cu (µg/L)	Cd (µg/L)	Pb (µg/L)	Ca (mg/L)	Mg (mg/L)
MO	2.77 ± 0.19	2.11 ± 0.17	0.09 ± 0.01	3.98 ±1.45	34.11 ± 0.81	0.00 ± 0.00
TER1	5.69 ± 0.28	3.19 ± 0.26	0.10 ± 0.01	4.71 ± 0.41	27.59 ± 0.35	1.19 ± 0.09
TER2	4.96 ± 0.16	1.81 ± 0.18	0.09 ± 0.01	1.83 ± 0.34	81.70 ± 1.43	9.39 ± 0.20
LRV	0.90 ± 0.05	0.61 ± 0.10	0.02 ± 0.00	0.16 ± 0.06	52.74 ± 1.76	12.88 ± 0.11
OHZ	0.96 ± 0.09	0.46 ± 0.13	0.04 ± 0.00	0.22 ± 0.01	53.72 ± 0.31	2.01 ± 0.06
ZW4	0.87 ± 0.09	0.71 ± 0.08	0.01 ± 0.00	0.05 ± 0.00	89.36 ± 0.31	2.87 ± 0.26
OM3	1.98 ± 0.05	0.54 ± 0.03	0.02 ± 0.00	0.65 ± 0.23	68.63 ± 0.11	2.98 ± 0.03
OM2	1.61 ± 0.07	0.78 ± 0.03	0.06 ± 0.00	0.49 ± 0.09	50.26 ± 0.34	1.72 ± 0.02
KNO 15	2.04 ± 0.10	1.60 ± 0.29	0.06 ± 0.01	1.63 ± 0.45	55.44 ± 0.47	4.80 ± 0.03
KNO 17	5.28 ± 0.00	1.31 ± 0.21	0.06 ± 0.00	0.59 ± 0.20	95.03 ± 0.21	11.99 ± 0.17
KNO 52	2.29 ± 0.14	2.56 ± 2.49	0.06 ± 0.00	1.54 ± 0.17	74.24 ± 4.21	3.86 ± 0.22

Table S5.91: Metal and organic carbon concentration in the sediment of different lakes. Samples were taken in April 2009. Values represent mean values ± standard deviation.

	Cd (mg Cd/kg)	Ni (mg Ni/kg)	Cu (mg Cu/kg)	Zn (mg Zn/kg)	Pb (mg Pb/kg)	Organic Carbon
MO	1.70 ± 0.11	12.98 ± 0.10	39.59 ± 8.20	203.38 ± 17.88	53.54 ± 3.31	0.95 ± 0.00
TER1	1.49 ± 0.04	12.64 ± 0.47	11.26 ± 0.41	42.45 ± 0.56	31.35 ± 1.57	0.96 ± 0.00
TER2	1.61 ± 0.03	16.99 ± 0.86	13.60 ± 0.16	52.23 ± 1.82	39.05 ± 0.92	0.95 ± 0.00
LRV	3.25 ± 0.20	16.87 ± 0.57	14.12 ± 0.37	57.46 ± 1.25	60.69 ± 3.28	0.88 ± 0.00
OHZ	1.23 ± 0.37	9.05 ± 1.21	12.16 ± 0.98	36.09 ± 2.88	40.14 ± 4.48	0.80 ± 0.00
ZW4	2.52 ± 0.14	13.41 ± 1.46	19.90 ± 2.55	94.15 ± 8.72	51.81 ± 2.83	0.92 ± 0.00
OM3	1.14 ± 0.25	3.62 ± 0.29	5.11 ± 0.50	38.45 ± 1.08	42.84 ± 18.22	0.98 ± 0.00
OM2	2.31 ± 0.41	12.79 ± 0.89	23.39 ± 2.11	143.42 ± 15.66	74.82 ± 7.12	0.91 ± 0.00
KNO15	1.21 ± 0.03	0.61 ± 0.29	0.99 ± 0.12	6.99 ± 0.78	15.99 ± 0.60	0.99 ± 0.00
KNO17	1.85 ± 1.85	3.57 ± 0.32	4.89 ± 4.89	24.52 ± 24.52	29.05 ± 29.05	0.96 ± 0.01
KNO52	1.44 ± 1.44	2.35 ± 0.50	1.93 ± 1.93	9.99 ± 9.99	19.53 ± 19.53	0.98 ± 0.00

Table S5.92: Biotic parameters in the different lakes. Fish abundance (scored in categories between 0 and 4, see Material and Method chapter 5) and prevalence of parasites (%) in the different lakes.

Pond	Fish	Parasites (prevalence %)			
	Abundance	*Amoebidium*	*Binucleata*	*Pasteuria*	*Vorticella*
KNO15	0.00	0.00	27.58	57.44	50.60
KNO17	1.00	1.25	59.17	63.46	17.74
KNO52	0.00	0.17	0.17	0.03	0.00
LRV	3.00	0.00	0.03	0.06	0.26
MO	0.00	0.00	25.00	6.58	67.50
OHZ	4.00	0.00	5.13	0.16	1.56
OM2	3.00	0.00	0.00	4.06	3.75
OM3	3.00	0.05	0.00	0.00	0.00
TER1	0.00	0.00	0.00	0.83	9.38
TER2	0.00	0.13	0.00	0.00	0.00
ZW4	4.00	0.00	0.00	0.04	0.00

Table S5.93: Land use characteristics of the different lakes, i.e. shortest distance to nearest crop field, percentage arable land, pastures and heterogeneous agricultural activities in a 200m radius.

Pond	Distance to cropfield (m)	Arable land (%)	Pastures (%)	Heterogeneous agricultural activities (%)
KNO15	611.12	0.00	84.25	0.00
KNO17	78.78	27.04	0.00	72.96
KNO52	947.97	0.00	70.08	0.00
LRV	278.42	0.00	24.11	17.29
MO	7.54	97.74	0.00	0.00
OHZ	488.55	0.00	27.17	30.75
OM2	34.38	29.78	0.00	0.00
OM3	49.41	17.16	0.00	0.00
TER1	52.22	7.40	0.00	92.60
TER2	121.96	31.42	0.00	68.58
ZW4	399.38	0.00	0.00	0.00

Table S5.94: Non parametric Spearman rank correlation coefficients between total reproduction in control treatment, Cd treatment, Cd tolerance and habitat characteristics. An asterisk indicates a significant correlation. Fish abundance (scored in categories between 0 and 4, see Material and Method chapter 5) and prevalence of parasites (%) were determined in the different lakes. Land use is determined by shortest distance to nearest crop field, and percentage arable land, pastures and heterogeneous agricultural activities in a 200m radius. Ranking of metals were determined by the summation of the ranks of each metal per population.

	Habitat characteristics	Total reproduction (0 µg Cd/L)	Total reproduction (5 µg Cd/L)	Total reproduction (Tolerance)
Fish abundance	Fish abundance	0.09	-0.10	-0.19
Parasite prevalence (%)	*Amoebidium* prevalence (%)	0.05	-0.53	-0.53
	Binucleata prevalence (%)	0.41	-0.25	-0.57
	Pasteuria prevalence (%)	0.50	-0.21	-0.54
	Vorticella prevalence (%)	0.45	0.37	-0.10
Land use	Distance to crop field (m)	-0.23	0.14	0.27
	Arable land (%)	0.11	0.05	-0.14
	Pastures (%)	0.17	0.29	0.18
	Heterogeneous agriculture (%)	-0.16	-0.43	-0.18
Metal concentrations in sediment	Cd (mg/kg)	0.22	0.15	-0.17
	Ni (mg/kg)	-0.07	-0.03	-0.06
	Cu (mg/kg)	0.05	0.21	-0.04
	Zn (mg/kg)	0.09	0.24	-0.03
	Pb (mg/kg)	0.21	0.08	-0.16
Metal concentrations in water	Ni (µg/L)	-0.22	-0.33	-0.06
	Cu (µg/L)	-0.38	0.27	0.52
	Cd (µg/L)	-0.11	-0.06	0.06
	Pb (µg/L)	-0.16	0.40	0.48
	Ca (mg/L)	-0.33	-0.67*	-0.42
	Mg (mg/L)	0.29	-0.40	-0.52
Ranking of metals	Metal ranking water	-0.18	0.16	0.19
	Metal ranking sediment	-0.02	0.06	-0.08

Table S5.95: Non parametric Spearman rank correlation coefficients between population growth rate in control treatment, Cd treatment, Cd tolerance and habitat characteristics. An asterisk indicates a significant correlation. Fish abundance (scored in categories between 0 and 4, see Material and Method chapter 5) and prevalence of parasites (%) were determined in the different lakes. Land use is determined by shortest distance to nearest crop field, and percentage arable land, pastures and heterogeneous agricultural activities in a 200m radius. Ranking of metals were determined by the summation of the ranks of each metal per population.

	Habitat characteristics	Population growth rate (0 µg Cd/L)	Population growth rate (5 µg Cd/L)	Population growth rate (Tolerance)
Fish presence	Fish abundance	0.02	-0.16	-0.49
Parasite prevalence (%)	*Amoebidium* prevalence (%)	0.14	-0.22	-0.35
	Binucleata prevalence (%)	0.34	0.03	-0.05
	Pasteuria prevalence (%)	0.54	0.11	-0.11
	Vorticella prevalence (%)	0.29	0.39	0.52
Land use	Distance to crop field (m)	0.09	0.20	-0.01
	Arable land (%)	-0.14	0.01	0.36
	Pastures (%)	0.41	0.43	0.25
	Heterogeneous agriculture (%)	-0.43	-0.45	-0.05
Metal concentrations in sediment	Cd (mg/kg)	0.18	0.23	-0.13
	Ni (mg/kg)	-0.49	-0.23	0.04
	Cu (mg/kg)	-0.28	0.05	0.30
	Zn (mg/kg)	-0.13	0.15	0.31
	Pb (mg/kg)	-0.02	0.04	0.00
Metal concentrations in water	Ni (µg/L)	-0.30	-0.30	0.09
	Cu (µg/L)	-0.23	0.21	0.57
	Cd (µg/L)	-0.33	-0.05	0.56
	Pb (µg/L)	-0.24	0.22	0.70*
	Ca (mg/L)	-0.09	-0.53	-0.88*
	Mg (mg/L)	0.22	-0.17	-0.38
Ranking of metals	Metal ranking water	-0.23	0.01	0.58
	Metal ranking sediment	-0.12	-0.07	0.03

Table S5.96: Non parameteric Spearman rank correlation coefficients between reproduction at first brood in control treatment, Cd treatment, Cd tolerance and habitat characteristics. An asterisk indicates a significant correlation. Fish abundance (scored in categories between 0 and 4, see Material and Method chapter 5) and prevalence of parasites (%) were determined in the different lakes. Land use is determined by shortest distance to nearest crop field, and percentage arable land, pastures and heterogeneous agricultural activities in a 200m radius. Ranking of metals were determined by the summation of the ranks of each metal per population.

Habitat characteristics		Reproduction at first brood (0 µg Cd/L)	Reproduction at first brood (5 µg Cd/L)	Reproduction at first brood (Tolerance)
Fish presence	Fish abundance	0.23	0.11	0.00
Parasite prevalence (%)	*Amoebidium* prevalence (%)	-0.03	-0.01	-0.23
	Binucleata prevalence (%)	0.24	0.13	-0.36
	Pasteuria prevalence (%)	0.37	0.15	-0.32
	Vorticella prevalence (%)	0.21	0.15	-0.21
Land use	Distance to crop field (m)	0.14	-0.18	-0.48
	Arable land (%)	-0.21	-0.01	0.18
	Pastures (%)	0.37	-0.01	-0.41
	Heterogeneous agriculture (%)	-0.07	0.11	0.21
Metal concentrations in sediment	Cd (mg/kg)	0.07	0.65*	0.54
	Ni (mg/kg)	-0.12	0.40	0.78*
	Cu (mg/kg)	-0.07	0.28	0.40
	Zn (mg/kg)	-0.08	0.25	0.40
	Pb (mg/kg)	0.07	0.42	0.63*
Metal concentrations in water	Ni (µg/L)	-0.31	-0.10	0.14
	Cu (µg/L)	-0.30	-0.06	-0.02
	Cd (µg/L)	-0.05	0.05	0.21
	Pb (µg/L)	-0.22	0.01	0.05
	Ca (mg/L)	-0.38	-0.45	-0.18
	Mg (mg/L)	0.04	0.25	0.23
Ranking of metals	Metal ranking water	-0.23	-0.04	0.15
	Metal ranking sediment	-0.09	0.33	0.76*

Table S5.97: Non parametric Spearman rank correlation coefficients between maturation rate in control treatment, Cd treatment, Cd tolerance and habitat characteristics. An asterisk indicates a significant correlation. Fish abundance (scored in categories between 0 and 4, see Material and Method chapter 5) and prevalence of parasites (%) were determined in the different lakes. Land use is determined by shortest distance to nearest crop field, and percentage arable land, pastures and heterogeneous agricultural activities in a 200m radius. Ranking of metals were determined by the summation of the ranks of each metal per population.

Habitat characteristics		Maturation rate (0 µg Cd/L)	Maturation rate (5 µg Cd/L)	Maturation rate (Tolerance)
Fish presence	Fish abundance	0.39	-0.02	-0.52
Parasite prevalence (%)	*Amoebidium* prevalence (%)	0.00	0.16	0.27
	Binucleata prevalence (%)	-0.40	0.10	0.61*
	Pasteuria prevalence (%)	-0.24	0.41	0.63*
	Vorticella prevalence (%)	-0.70*	-0.15	0.62*
Land use	Distance to crop field (m)	0.22	-0.32	-0.44
	Arable land (%)	-0.56	-0.38	0.34
	Pastures (%)	-0.01	-0.10	-0.07
	Heterogeneous agriculture (%)	-0.18	0.35	0.51
Metal concentrations in sediment	Cd (mg/kg)	0.55	0.19	-0.33
	Ni (mg/kg)	0.12	0.02	-0.08
	Cu (mg/kg)	-0.34	-0.38	0.05
	Zn (mg/kg)	-0.30	-0.33	0.03
	Pb (mg/kg)	0.17	-0.04	-0.27
Metal concentrations in water	Ni (µg/L)	-0.29	0.30	0.61*
	Cu (µg/L)	-0.34	-0.08	0.35
	Cd (µg/L)	-0.68*	-0.06	0.66*
	Pb (µg/L)	-0.60	-0.10	0.53
	Ca (mg/L)	0.58	0.12	-0.33
	Mg (mg/L)	0.45	0.39	0.03
Ranking of metals	Metal ranking water	-0.55	-0.14	0.57
	Metal ranking sediment	0.09	-0.05	-0.19

Table S5.98: Non parametric Spearman rank correlation coefficients between genetic coefficient of variation and broad sense heritability (H^2) in control, cd treatment and Cd tolerance of total reproduction and habitat characteristics. An asterisk indicates a significant correlation ($p<0.05$). Fish abundance (scored in categories between 0 and 4, see Material and Method chapter 5) and prevalence of parasites (%) were determined in the different lakes. Land use is determined by shortest distance to nearest crop field, and percentage arable land, pastures and heterogeneous agricultural activities in a 200m radius. Ranking of metals were determined by the summation of the ranks of each metal per population.

Habitat characteristics		CV_G total reproduction			H^2 total reproduction		
		0 µg Cd/L	5 µg Cd/L	Cd tolerance	0 µg Cd/L	5 µg Cd/L	Cd tolerance
Fish presence	Fish abundance	-0.10	0.23	-0.01	0.00	0.37	0.24
Parasite prevalence (%)	*Amoebidium* prevalence (%)	0.01	0.53	0.49	0.15	0.30	0.04
	Binucleata prevalence (%)	-0.39	0.21	0.42	-0.22	0.14	0.16
	Pasteuria prevalence (%)	-0.39	0.17	0.47	-0.23	0.02	0.29
	Vorticella prevalence (%)	-0.51	-0.46	-0.12	-0.43	-0.27	-0.03
Land use	Distance to crop field (m)	0.06	-0.31	-0.08	-0.02	-0.38	0.13
	Arable land (%)	-0.25	-0.18	-0.18	-0.23	-0.07	-0.51
	Pastures (%)	-0.19	-0.43	-0.08	-0.24	-0.52	0.15
	Heterogeneous agriculture (%)	0.19	0.61*	0.59	0.18	0.37	0.38
Metal concentrations in sediment	Cd (mg/kg)	0.21	-0.02	-0.05	0.28	-0.13	0.03
	Ni (mg/kg)	0.12	0.14	0.07	0.05	0.05	-0.01
	Cu (mg/kg)	-0.12	-0.28	-0.24	-0.08	-0.14	-0.24
	Zn (mg/kg)	-0.08	-0.36	-0.33	-0.03	-0.23	-0.36
	Pb (mg/kg)	0.00	-0.05	-0.20	0.06	-0.04	-0.21
Metal concentrations in water	Ni (µg/L)	0.26	0.36	0.37	0.23	0.17	-0.01
	Cu (µg/L)	0.44	-0.36	-0.15	0.35	-0.41	-0.08
	Cd (µg/L)	0.04	-0.11	0.19	-0.02	-0.31	-0.06
	Pb (µg/L)	0.19	-0.39	-0.24	0.14	-0.25	-0.05
	Ca (mg/L)	0.11	0.57	0.39	0.08	0.40	-0.20
	Mg (mg/L)	-0.10	0.57	0.47	-0.10	0.24	0.05
Ranking of metals	Metal ranking water	0.15	-0.27	-0.04	0.17	-0.29	-0.12
	Metal ranking sediment	0.20	-0.05	-0.08	0.17	-0.10	-0.12

Table S5.99: Non parametric Spearman rank correlation coefficients between genetic coefficient of variation, broad sense heritability (H^2) in control, cd treatment and Cd tolerance of population growth rate and habitat characteristics. An asterisk indicates a significant correlation ($p<0.05$). Fish abundance (scored in categories between 0 and 4, see Material and Method chapter 5) and prevalence of parasites (%) were determined in the different lakes. Land use is determined by shortest distance to nearest crop field, and percentage arable land, pastures and heterogeneous agricultural activities in a 200m radius. Ranking of metals were determined by the summation of the ranks of each metal per population.

Habitat characteristics		CV_G population growth rate			H^2 population growth rate		
		0 µg Cd/L	5 µg Cd/L	Cd tolerance	0 µg Cd/L	5 µg Cd/L	Cd tolerance
Fish presence	Fish abundance	-0.20	0.21	0.09	-0.13	0.44	-0.01
Parasite prevalence (%)	*Amoebidium* prevalence (%)	0.07	0.19	0.43	0.03	0.19	0.54
	Binucleata prevalence (%)	-0.24	-0.21	0.10	-0.19	-0.34	0.38
	Pasteuria prevalence (%)	-0.15	-0.22	0.13	-0.14	-0.44	0.44
	Vorticella prevalence (%)	-0.40	-0.63*	-0.47	-0.29	-0.83	-0.20
Land use	Distance to crop field (m)	-0.35	-0.22	-0.17	-0.39	-0.18	-0.13
	Arable land (%)	-0.17	-0.19	-0.28	-0.12	-0.30	-0.18
	Pastures (%)	-0.34	-0.48	-0.16	-0.33	-0.48	-0.08
	Heterogeneous agriculture (%)	0.61*	0.51	0.45	0.54	0.31	0.43
Metal concentrations in sediment	Cd (mg/kg)	0.29	-0.03	-0.12	0.23	0.43	-0.46
	Ni (mg/kg)	0.41	0.34	-0.10	0.32	0.41	-0.37
	Cu (mg/kg)	-0.05	-0.16	-0.56	0.02	-0.15	-0.58
	Zn (mg/kg)	-0.05	-0.24	-0.60	0.04	-0.22	-0.59
	Pb (mg/kg)	0.16	0.04	-0.22	0.21	0.29	-0.44
Metal concentrations in water	Ni (µg/L)	0.54	0.31	0.30	0.47	0.04	0.38
	Cu (µg/L)	0.40	-0.21	-0.28	0.39	-0.33	-0.24
	Cd (µg/L)	0.36	-0.06	-0.22	0.37	-0.44	-0.05
	Pb (µg/L)	0.29	-0.29	-0.36	0.36	-0.44	-0.29
	Ca (mg/L)	-0.16	0.57	0.47	-0.39	0.45	0.54
	Mg (mg/L)	0.15	0.31	0.68*	-0.03	0.52	0.43
Ranking of metals	Metal ranking water	0.14	-0.28	-0.04	0.15	-0.44	-0.02
	Metal ranking sediment	0.27	0.12	-0.37	0.31	0.12	-0.56

Table S5.100: Non parametric Spearman rank correlation coefficients between genetic coefficient of variation, broad sense heritability (H^2) in control, cd treatment and Cd tolerance of reproduction at first brood rate and habitat characteristics. An asterisk indicates a significant correlation ($p<0.05$). Fish abundance (scored in categories between 0 and 4, see Material and Method chapter 5) and prevalence of parasites (%) were determined in the different lakes. Land use is determined by shortest distance to nearest crop field, and percentage arable land, pastures and heterogeneous agricultural activities in a 200m radius. Ranking of metals were determined by the summation of the ranks of each metal per population.

Habitat characteristics		CV_G reproduction at first brood			H^2 reproduction at first brood		
		0 µg Cd/L	5 µg Cd/L	Cd tolerance	0 µg Cd/L	5 µg Cd/L	Cd tolerance
Fish presence	Fish abundance	0.25	-0.50	-0.58	0.14	-0.65	-0.54
Parasite prevalence (%)	*Amoebidium* prevalence (%)	-0.62*	0.22	0.36	-0.51	0.48	0.57
	Binucleata prevalence (%)	-0.42	-0.08	0.44	-0.15	0.20	0.81*
	Pasteuria prevalence (%)	-0.44	-0.20	0.34	-0.27	0.02	0.67*
	Vorticella prevalence (%)	0.15	-0.19	0.30	0.46	-0.07	0.63*
Land use	Distance to crop field (m)	-0.21	-0.36	-0.25	-0.24	-0.25	-0.24
	Arable land (%)	0.26	0.22	0.27	0.52	0.31	0.51
	Pastures (%)	-0.30	-0.33	-0.13	-0.26	-0.26	-0.04
	Heterogeneous agriculture (%)	-0.37	0.51	0.79*	-0.43	0.58	0.43
Metal concentrations in sediment	Cd (mg/kg)	-0.08	-0.25	-0.45	-0.10	-0.25	-0.34
	Ni (mg/kg)	0.32	0.03	0.06	0.25	-0.05	-0.16
	Cu (mg/kg)	0.48	-0.16	-0.03	0.65*	-0.14	0.08
	Zn (mg/kg)	0.45	-0.14	-0.15	0.63*	-0.14	0.04
	Pb (mg/kg)	0.31	-0.20	-0.45	0.30	-0.33	-0.38
Metal concentrations in water	Ni (µg/L)	-0.32	0.71*	0.79*	-0.31	0.82*	0.57
	Cu (µg/L)	-0.21	0.55	0.50	-0.16	0.64*	0.25
	Cd (µg/L)	-0.17	0.42	0.76*	-0.04	0.56	0.58
	Pb (µg/L)	0.09	0.50	0.54	0.20	0.48	0.32
	Ca (mg/L)	-0.09	-0.04	-0.08	-0.20	0.14	0.10
	Mg (mg/L)	-0.50	0.12	0.13	-0.53	0.23	0.24
Ranking of metals	Metal ranking water	-0.17	0.63*	0.73*	-0.12	0.66*	0.66*
	Metal ranking sediment	0.36	-0.11	-0.32	0.30	-0.16	-0.38

Table S5.101: Non parametric Spearman rank correlation coefficients between genetic coefficient of variation, broad sense heritability (H^2) in control, cd treatment and Cd tolerance of maturation rate and habitat characteristics. An asterisk indicates a significant correlation ($p<0.05$). Fish abundance (scored in categories between 0 and 4, see Material and Method chapter 5) and prevalence of parasites (%) were determined in the different lakes. Land use is determined by shortest distance to nearest crop field, and percentage arable land, pastures and heterogeneous agricultural activities in a 200m radius. Ranking of metals were determined by the summation of the ranks of each metal per population.

Habitat characteristics		CV_G maturation rate			H^2 maturation rate		
		0 µg Cd/L	5 µg Cd/L	Cd tolerance	0 µg Cd/L	5 µg Cd/L	Cd tolerance
Fish presence	Fish abundance	0.06	-0.17	0.01	0.02	-0.15	-0.03
Parasite prevalence (%)	*Amoebidium* prevalence (%)	0.19	0.07	0.14	0.09	0.11	0.05
	Binucleata prevalence (%)	0.38	0.05	0.08	0.38	0.34	0.26
	Pasteuria prevalence (%)	0.57	0.12	0.16	0.62	0.44	0.55
	Vorticella prevalence (%)	0.23	0.07	-0.04	0.33	0.41	0.25
Land use	Distance to crop field (m)	-0.41	0.05	0.57*	-0.07	0.04	0.49
	Arable land (%)	-0.06	0.03	-0.22	-0.23	0.11	-0.32
	Pastures (%)	-0.17	0.10	0.47	0.13	0.16	0.62
	Heterogeneous agriculture (%)	0.22	-0.13	-0.48	0.04	-0.24	-0.46
Metal concentrations in sediment	Cd (mg/kg)	0.15	0.69	0.49	0.14	0.53	0.26
	Ni (mg/kg)	0.03	0.27	-0.20	-0.12	0.08	-0.37
	Cu (mg/kg)	0.00	0.22	-0.10	-0.14	0.24	-0.25
	Zn (mg/kg)	0.04	0.28	-0.02	-0.13	0.31	-0.15
	Pb (mg/kg)	0.10	0.31	0.01	-0.14	0.17	-0.15
Metal concentrations in water	Ni (µg/L)	0.19	-0.03	-0.38	0.01	-0.09	-0.36
	Cu (µg/L)	-0.17	0.17	-0.01	-0.13	0.06	-0.12
	Cd (µg/L)	0.07	0.08	-0.31	-0.10	0.04	-0.25
	Pb (µg/L)	-0.04	0.00	-0.34	-0.06	-0.02	-0.33
	Ca (mg/L)	-0.01	-0.04	0.30	0.06	0.05	0.20
	Mg (mg/L)	0.10	0.25	0.21	0.14	0.14	0.16
Ranking of metals	Metal ranking water	-0.14	0.19	-0.32	-0.18	-0.18	-0.31
	Metal ranking sediment	0.15	0.30	-0.07	-0.02	0.13	-0.22

Table 5.102 General Linear Model for population and clone effect for Cd tolerance of total reproduction (R_0). An asterisk indicates a significant effect.

Fitness traits	Likihood ratio Chi-square effect	df	p-value
Intercept	369.61	1	<0.01*
Clone(Population)	186.53	109	<0.01*
Population	68.16	10	<0.01*

Table 5.103: General Linear Model for population and clone effect for Cd tolerance of population growth rate (r_m). An asterisk indicates a significant effect.

Fitness traits	Likihood ratio Chi-square effect	df	p-value
Intercept	1088.44	1	<0.01*
Clone(Population)	410.75	109	<0.01*
Population	125.72	10	<0.01*

Table 5.104: General Linear Model for population and clone effect for Cd tolerance of reproduction at first brood. An asterisk indicates a significant effect.

Fitness traits	Likihood ratio Chi-square effect	df	p-value
Intercept	156.26	1	<0.01
Clone(Population)	130.32	105	0.05*
Population	9.45	10	0.49

Table 5.105: General Linear Model for population and clone effect for Cd tolerance of maturation rate. An asterisk indicates a significant effect.

Fitness traits	Likihood ratio Chi-square effect	df	p-value
Intercept	1240.48	1	<0.01*
Clone(Population)	152.63	105	<0.01*
Population	27.10	10	<0.01*

Table 5.106: General Linear Model for population and clone effect of total reproduction (R_0) in control treatment. An asterisk indicates a significant effect.

Fitness traits	Likihood ratio Chi-square effect	df	p-value
Intercept	1002.59	1	<0.01*
Clone(Population)	230.57	114	0.01*
Population	30.94	10	<0.01*

Table 5.107: General Linear Model for population and clone effect of total reproduction (R_0) in Cd treatment. An asterisk indicates a significant effect.

Fitness traits	Likihood ratio Chi-square effect	df	p-value
Intercept	981.45	1	<0.01*
Clone(Population)	395.73	114	<0.01*
Population	100.06	10	<0.01*

Table 5.108: General Linear Model for population and clone effect of population growth rate (r_m) in control treatment. An asterisk indicates a significant effect.

Fitness traits	Likihood ratio Chi-square effect	Df	p-value
Intercept	1037.77	1	<0.01*
Clone(Population)	218.57	113	<0.01*
Population	44.09	10	<0.01*

Table 5.109: General Linear Model for population and clone effect of population growth rate (r_m) in Cd treatment. An asterisk indicates a significant effect.

Fitness traits	Likihood ratio Chi-square effect	Df	p-value
Intercept	995.63	1	<0.01*
Clone(Population)	379.35	114	<0.01*
Population	111.19	10	<0.01*

Table 5.110: General Linear Model for population and clone effect of reproduction at first brood in control treatment. An asterisk indicates a significant effect.

Fitness traits	Likihood ratio Chi-square effect	Df	p-value
Intercept	1410.36	1	<0.01*
Clone(Population)	218.86	112	<0.01*
Population	39.70	10	<0.01*

Table 5.111: General Linear Model for population and clone effect of reproduction at first brood in Cd treatment. An asterisk indicates a significant effect.

Fitness traits	Likihood ratio Chi-square effect	Df	p-value
Intercept	1277.98	1	<0.01*
Clone(Population)	284.65	109	<0.01*
Population	92.19	10	<0.01*

Table 5.112: General Linear Model for population and clone effect of maturation rate in control treatment. An asterisk indicates a significant effect.

Fitness traits	Likihood ratio Chi-square effect	Df	p-value
Intercept	282.16	1	<0.01*
Clone(Population)	119.74	112	0.59
Population	8.41	10	0.29

Table 5.113: General Linear Model for population and clone effect of maturation rate in Cd treatment. An asterisk indicates a significant effect.

Fitness traits	Likihood ratio Chi-square effect	Df	p-value
Intercept	208.59	1	<0.01*
Clone(Population)	112.73	109	0.38
Population	8.98	10	0.53

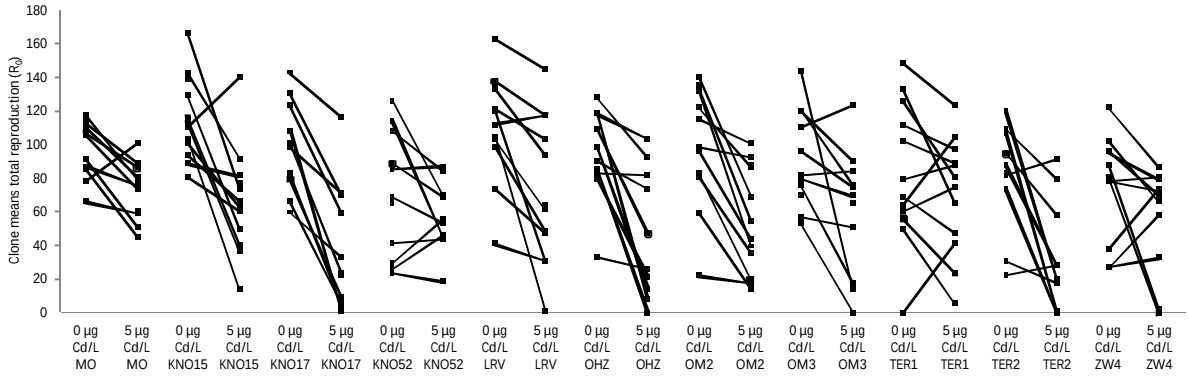

Figure S5.1: Clone means of total reproduction (R_0) for different populations in control and Cd treatment.

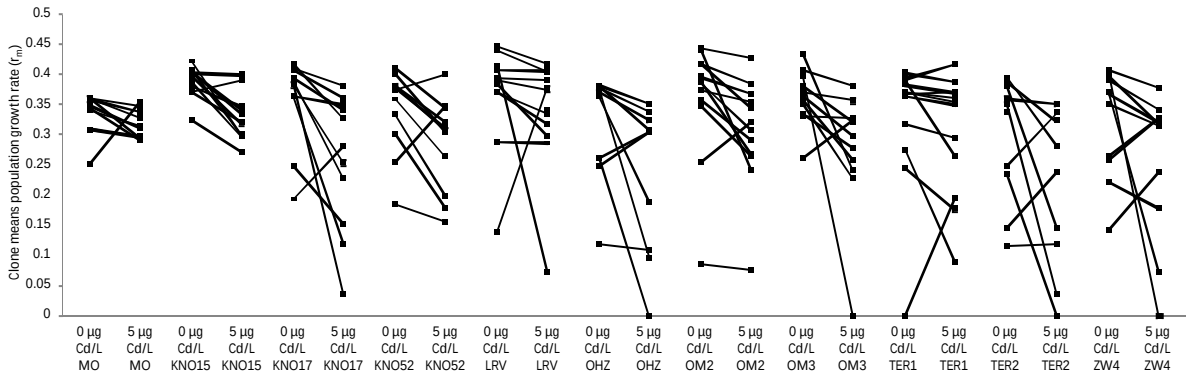

Figure S5.2: Clone means of population growth rate for different populations in control and Cd treatment.

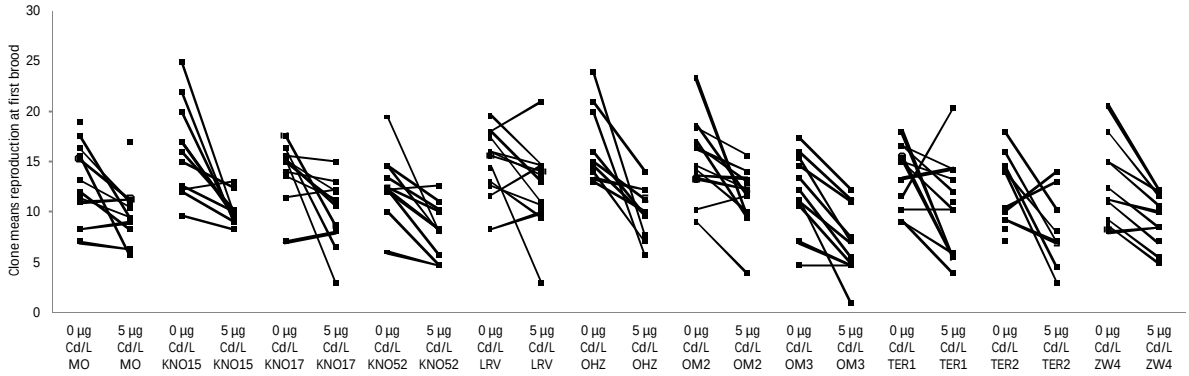

Figure S5.3: Clone means of reproduction at first brood for different populations in control and Cd treatment.

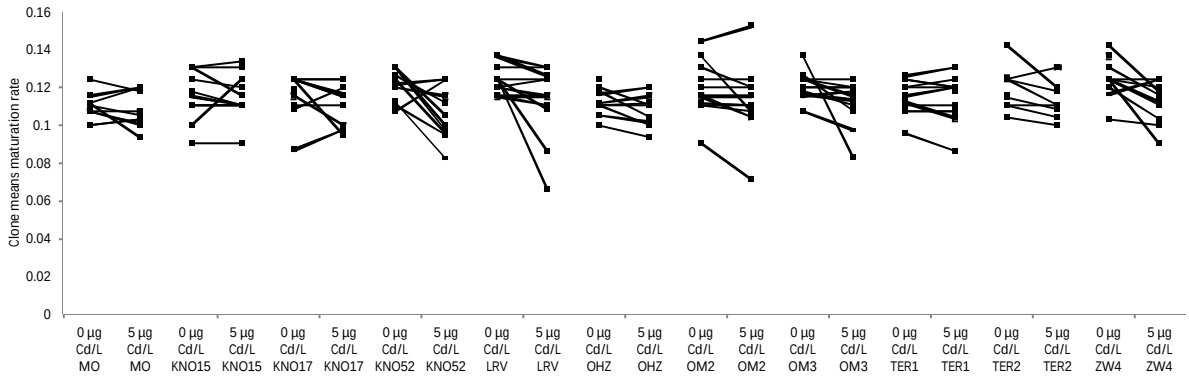

Figure S5.4: Clone means of maturation rate for different populations in control and Cd treatment.

Table S6.1: Physico-chemical characteristics of test media during Cd experiment. Values represent mean ± standard deviation. NM is new medium. OM is old medium.

Nominal Cd concentration (µg Cd/L)		DOC (mg C/L)	pH	Cd concentration (µg Cd/L)	Mean Cd concentration (µg Cd/L)
0	NM	4.1	7.69	<0.1	<0.1
	OM	5.21 ±0.71	7.53 ± 0.12	<0.1	
2.2	NM	4.89	7.69	2.05 ± 0.08	2.02 ± 0.04
	OM	5.93 ± 0.63	7.72 ± 0.21	1.99 ± 0.06	
4.6	NM	4.73	7.62	5.12 ± 0.15	4.70 ± 0.60
	OM	5.72 ±0.33	7.70 ± 0.31	4.28 ± 0.60	
10	NM	4.83	7.61	9.32 ± 0.89	9.13 ± 0.89
	OM	5.89 ± 0.71	7.68 ± 0.17	8.93 ± 1.21	
22	NM	4.52	7.52	20.04 ± 0.54	17.83 ± 3.13
	OM	5.12 ± 1.512	7.83 ± 0.31	15.61 ± 1.07	

Table S6.2: Physico-chemical characteristics of test media during micro-evolutionary experiment. Values represent mean ± standard deviation. NM is new medium. OM is old medium.

Nominal Cd concentration (µg Cd/L)		DOC (mg C/L)	pH	Cd concentration (µg Cd/L)	Mean Cd concentration (µg Cd/L)
0	NM	4.62	7.67	<0.1	<0.1
	OM	5.45 ±0.64	7.58 ± 0.22	<0.1	
2.2	NM	4.84	7.65	2.61 ± 0.35	2.11 ± 0.71
	OM	5.83 ± 0.52	7.70 ± 0.31	1.61 ± 0.15	
4.6	NM	4.86	7.62	5.10 ± 0.14	4.70 ± 0.56
	OM	5.99 ±0.30	7.73 ± 0.25	4.31 ± 0.18	
10	NM	4.69	7.65	10.05 ± 0.18	9.21 ± 1.20
	OM	5.97 ± 0.67	7.76 ± 0.27	8.36 ± 0.26	
22	NM	4.76	7.66	23.19 ± 1.24	20.77 ± 3.41
	OM	5.89 ± 1.32	7.86 ± 0.27	18.37 ± 1.51	

Table S6.3: Values of total reproduction of start population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	76		150	93	0	49	101	90	123	119	82	76
2	118	126	144	33	153	17		95	128		115	118
3	87		138	132		140	27	85		119	102	87

Table S6.4: Values of population growth rate of start population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.33		0.47	0.32	0.00	0.36	0.41	0.38	0.39	0.45	0.41	0.33
2	0.39	0.39	0.43	0.42	0.42	0.31		0.39	0.40		0.42	0.39
3	0.38		0.41	0.37		0.53	0.36	0.33		0.39	0.41	0.38

Table S6.5: Values of total reproduction of start population in 2.2 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	90		104	89	41	93	92	82	96	26	0	90
2	89	48	109	0	114	68		72	116		11	89
3	26		3	105		135	79	76		84	0	26

Table S6.6: Values of population growth rate of start population in 2.2 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.35		0.47	0.35	0.36	0.32	0.38	0.35	0.36	0.31	0.00	0.35
2	0.36	0.33	0.37	0.00	0.40	0.33		0.33	0.34		0.30	0.36
3	0.34		0.14	0.35		0.48	0.36	0.33		0.36	0.00	0.34

Table S6.7: Values of total reproduction of start population in 4.6 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	78		0	52	137	4	112	116	0	6	2	78
2	68	0	0	0	126	0		89	0		11	68
3	26		10	0		15	23	84		33	0	26

Table S6.8: Values of population growth rate of start population in 4.6 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.34		0.00	0.31	0.37	0.11	0.39	0.33	0.00	0.26	0.06	0.34
2	0.38	0.00	0.00	0.00	0.39	0.00		0.37	0.00		0.30	0.38
3	0.34		0.15	0.00		0.34	0.35	0.33		0.33	0.00	0.34

Table S6.9: Values of total reproduction of start population in 10 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	10		19	69	38	0	69	91	0	0	0	10
2	0	43	0	0	91	0		98	80		0	0
3	4		9	52		17	0	82		0	0	4

Table S6.10: Values of population growth rate of start population in 10 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.26		0.30	0.32	0.31	0.00	0.35	0.32	0.00	0.00	0.00	0.26
2	0.00	0.29	0.00	0.00	0.37	0.00		0.33	0.29		0.00	0.00
3	0.17		0.27	0.30		0.31	0.00	0.32		0.00	0.00	0.17

Table S6.11: Values of total reproduction of start population in 22 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	61		4	0	0	0	0	5	0	0	0	61
2	0	0	6	0	0	0		12	0		0	0
3	0		6	0		0	25	11		0	0	0

Table S6.12: Values of population growth rate of start population in 22 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12
1	0.33		0.15	0.00	0.00	0.00	0.00	0.18	0.00	0.00	0.00	0.33
2	0.00	0.00	0.22	0.00	0.00	0.00		0.26	0.00		0.00	0.00
3	0.00		0.22	0.00		0.00	0.38	0.21		0.00	0.00	0.00

Table S6.13: Values of total reproduction of Control-evolved population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	80	132	174	117	100	72		77	94	109	93	121	69	86	
2	128	96	84	124	53	91	0	97	0	87	61		92	100	69
3	142	142	0	117	95	69	86	73	0	78	0	84	0	111	74

Table S6.14: Values of population growth rate of Control-evolved population in control treatment.

Clone / Replicat	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.36	0.46	0.46	0.36	0.38	0.37		0.37	0.41	0.37	0.36	0.46	0.39	0.40	
2	0.38	0.41	0.35	0.44	0.34	0.38	0.00	0.41	0.00	0.39	0.35		0.39	0.43	0.39
3	0.38	0.44	0.00	0.39	0.38	0.40	0.35	0.34	0.00	0.42	0.00	0.40	0.00	0.38	0.42

Table S6.15: Values of total reproduction of Control-evolved population in 2.2 µg Cd/L.

Clone / Replicat	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	79	131	168	0	94	85		89	89	78	76	53	87	110	
2	110	116	106	90	73	60	62	92	76	104	57		59	109	79
3	109	120	91	88	94	65	88	136	48	70	76	86		95	75

Table S6.16: Values of population growth rate of Control-evolved population in 2.2 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.36	0.37	0.45	0.00	0.37	0.33		0.37	0.38	0.36	0.35	0.39	0.41	0.40	
2	0.39	0.42	0.39	0.41	0.31	0.32	0.35	0.33	0.31	0.38	0.35		0.30	0.40	0.38
3	0.39	0.41	0.38	0.35	0.33	0.37	0.32	0.40	0.30	0.31	0.40	0.34		0.34	0.40

Table S6.17: Values of total reproduction of Control-evolved population in 4.6 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	126	0	0	109	0	85		121	82	89	0	121	93	116	
2	120	109	115	0	87	0	85	28	57	92	64		87	64	96
3	116	118	72	120	60	34	87	87	44	83	98	106		107	87

Table S6.18: Values of population growth rate of Control-evolved population in 4.6 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.35	0.00	0.00	0.35	0.00	0.34		0.38	0.35	0.34	0.00	0.41	0.39	0.38	
2	0.36	0.37	0.39	0.00	0.33	0.00	0.34	0.33	0.32	0.38	0.34		0.38	0.32	0.41
3	0.36	0.38	0.39	0.35	0.35	0.33	0.33	0.33	0.31	0.33	0.38	0.33		0.37	0.41

Table S6.19: Values of total reproduction of Control-evolved population in 10 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0	0	0	9	16	0		81	62	38	31	146	76	34	
2	119	85	30	0	22	37	0	57	4	71	42		93	29	76
3	7	96	31	16	23	35	70	34	11	65	60	92		5	73

Table S6.20: Values of population growth rate of Control-evolved population in 10 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.00	0.00	0.00	0.24	0.30	0.00		0.36	0.33	0.31	0.36	0.44	0.39	0.31	
2	0.37	0.38	0.27	0.00	0.29	0.33	0.00	0.25	0.14	0.34	0.31		0.40	0.27	0.37
3	0.24	0.39	0.38	0.29	0.31	0.34	0.33	0.33	0.24	0.37	0.33	0.38		0.20	0.40

Table S6.21: Values of total reproduction of Control-evolved population in 22 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0	13	10	0	0	0	0	0	29	28	7	27	70	5	
2	0	0	3	0	0	2	23	13	5	30	0		78	0	86
3	89	19	26	29	5	5	12	0	0	46	42	30		29	81

Table S6.23: Values of population growth rate of Control-evolved population in 22 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.00	0.27	0.29	0.00	0.00	0.00	0.00	0.00	0.26	0.30	0.28	0.36	0.37	0.19	
2	0.00	0.00	0.10	0.00	0.00	0.08	0.30	0.20	0.16	0.32	0.00		0.35	0.00	0.41
3	0.32	0.33	0.36	0.31	0.18	0.18	0.21	0.00	0.00	0.30	0.32	0.37		0.00	0.42

Table S6.24: Values of total reproduction of 2.2 µg Cd/L -evolved population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	143			124	136	109	135	127	124	132	97	92	143	114	124
2		127		147		91	161	145	124	115	66	74	117	0	124
3				158	0	132	0	144	119		122		117	163	160

Table S6.25: Values of population growth rate of 2.2 µg Cd/L -evolved population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.47			0.44	0.41	0.38	0.35	0.37	0.43	0.43	0.39	0.37	0.36	0.39	0.41
2		0.46		0.42		0.37	0.39	0.46	0.39	0.40	0.36	0.35	0.38	0.00	0.43
3				0.40	0.00	0.38	0.00	0.41	0.43		0.40		0.37	0.40	0.41

Table S6.26: Values of total reproduction of 2.2 µg Cd/L -evolved population in 2.2 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	136			18	112	57	6	11	104	9	78	73	101	106	62
2		99		15	129	97	11	58	124	18	89	95	73	111	15
3				32	0	81	9	23	92	63	77	33	124	91	8

Table S6.27: Values of population growth rate of 2.2 µg Cd/L -evolved population in 2.2 µg Cd/L

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.45			0.36	0.36	0.35	0.22	0.28	0.38	0.16	0.28	0.33	0.33	0.38	0.29
2		0.41		0.30	0.39	0.37	0.30	0.38	0.39	0.31	0.35	0.37	0.37	0.38	0.33
3				0.31	0.00	0.30	0.23	0.31	0.36	0.34	0.34	0.28	0.38	0.36	0.26

Table S6.28: Values of total reproduction of 4.6 µg Cd/L -evolved population in control treatment

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	128		98	97	0	0	36	86		128	94		89	121	135
2	146		91		0		103		97	95	93		89	119	
3	136	0	134	132		105	96		90	103	111	84	33	24	106

Table S6.29: Values of population growth rate of 4.6 µg Cd/L -evolved population in control treatment

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.37		0.39	0.34	0.00	0.00	0.29	0.34		0.38	0.40		0.34	0.36	0.41
2	0.39		0.36		0.00		0.33		0.34	0.37	0.32		0.35	0.38	
3	0.38	0.00	0.40	0.41		0.34	0.33		0.36	0.39	0.38	0.32	0.36	0.16	0.36

Table S6.30: Values of total reproduction of 4.6 µg Cd/L -evolved population in 4.6 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		0	56	118	128	90	21	98		0	0		62	0	
2		0	44		108		0		109	0	0		77	0	
3	51	34	139	139		98	0		0	113	0	76	87	0	120

Table S6.31: Values of population growth rate of 4.6 µg Cd/L -evolved population in 4.6 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		0.00	0.30	0.34	0.35	0.31	0.23	0.35		0.00	0.00		0.33	0.00	
2		0.00	0.27		0.35		0.00		0.31	0.00	0.00		0.33	0.00	
3	0.32	0.30	0.37	0.36		0.31	0.00		0.00	0.33	0.00	0.32	0.35	0.00	0.36

Table S6.32: Values of total reproduction of 10 µg Cd/L -evolved population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	92	92	91	84	89	84	79	108	97	95	86	93	115	131	0
2	84	127	139	118	0	97	87	78	117	97	106	89	58	116	94
3		128	35	102	148	98	95	113	121	89	77	67	107	62	88

Table S6.33: Values of population growth rate of 10 µg Cd/L -evolved population in control treatment.

Clone / Replicat	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.35	0.32	0.34	0.36	0.36	0.34	0.39	0.34	0.35	0.39	0.34	0.37	0.39	0.39	0.00
2	0.31	0.39	0.41	0.36	0.00	0.36	0.34	0.33	0.35	0.37	0.38	0.35	0.27	0.38	0.35
3		0.34	0.36	0.37	0.40	0.36	0.28	0.34	0.35	0.37	0.31	0.36	0.37	0.29	0.34

Table S6.34: Values of total reproduction of 10 µg Cd/L -evolved population in 10 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	95	33	4	0	80	79	89	56	69	122	70	0	2	88	28
2	106	93	1	0		75	0	67	86	0	65	84	71	102	38
3		0	0	107	81	0	17	0	85	0	0	67	80		62

Table S6.35: Values of population growth rate of 10 µg Cd/L -evolved population in 10 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.35	0.34	0.07	0.00	0.31	0.30	0.38	0.29	0.33	0.35	0.31	0.00	0.09	0.35	0.26
2	0.32	0.31	0.00	0.00		0.31	0.00	0.31	0.33	0.00	0.33	0.33	0.36	0.35	0.28
3		0.00	0.00	0.35	0.37	0.00	0.31	0.00	0.33	0.00	0.00	0.29	0.35		0.31

Table S6.36: Values of total reproduction of 22 µg Cd/L -evolved population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0	78	90	82	119	41	89	93	80	69	96	83	88	101	70
2	0	69		130	0	73	71	87	93	107	112	102	26	96	90
3	0	122		72	121	87	97	106	81	124	82	115	56		94

Table S6.37: Values of population growth rate of 22 µg Cd/L -evolved population in control treatment.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.00	0.34	0.36	0.35	0.39	0.33	0.34	0.35	0.38	0.36	0.40	0.37	0.39	0.36	0.30
2	0.00	0.29		0.36	0.00	0.39	0.38	0.37	0.38	0.40	0.38	0.35	0.30	0.36	0.37
3	0.00	0.37		0.35	0.37	0.38	0.37	0.42	0.35	0.40	0.40	0.37	0.36		0.35

Table S6.38: Values of total reproduction of 22 µg Cd/L -evolved population in 22 µg Cd/L.

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	81	74	30	35	46	48	0	15	52	62	58	43	29	0	2
2	89	0		21	33	73	9	0	43	39	45	27	4		36
3	91	65		0	57	40	0	0	45	35	15	64	2	90	31

Table S6.39: Values of population growth rate 22 µg Cd/L -evolved population in 22 µg Cd/L

Clone / Replicate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.35	0.31	0.33	0.29	0.33	0.37	0.00	0.30	0.35	0.36	0.28	0.36	0.27	0.00	0.08
2	0.33	0.00		0.31	0.33	0.35	0.24	0.00	0.33	0.34	0.33	0.29	0.09		0.29
3	0.35	0.30		0.00	0.30	0.35	0.00	0.00	0.35	0.32	0.29	0.37	0.09	0.36	0.27

Table S6.40: Total ephippia production during micro-evolution experiment per aquaria per Cd-evolved population.

Cd-evolved population / Aquaria	0	2.2	4.6	10	22
1	509	289	271	223	267
2	313	386	301	298	332
3	590	304	366	191	383

Table S6.41: Results of Kruskall- Wallis test. P-values indicate significance p-level for differences between Cd-evolved populations per day of experiment

day of experiment	1	24	34	43	50	58	65	72	79	86	93	100	105
Kruskall-wallis p-value	1	1	0.53	0.5	0.41	0.66	0.07	0.13	0.74	0.13	0.22	0.04	0.03

Table S6.42: Results of Kruskall- Wallis test. P-values indicate significance p-level for differences between Cd-evolved populations per day of experiment

day of experiment	112	120	127	133	140	147	155	160	170	177	184
Kruskall-wallis p-value	0.38	0.03	0.07	0.59	0.16	0.1	0.07	0.04	0.31	0.73	0.12

S6.1. General Linear Models

A general Linear model can be seen as an extension of linair multiple regressions for a single dependent variable (response variable):

(Eq. S6.1)

Where y is the response variable and the z_i are the predictor (or explanatory) variables to predict the value of the response variable. The response variable (y) is now in function of n variables. The variables y and z represent observed variables, whereas α and β are constants to be estimated. In nested designs, the omitted effects are low-order effects. Nested effects are effects in which the nested variables never appear as main effects (variables).

S7.1. Materials and methods of 21-day life table experiment with monoclonal *Daphnia magna* laboratory populations

S7.1.1. General culture and exposure conditions

The maintenance and exposures of all clones of laboratory populations were performed at 20°C and under a light:dark cycle of 16h:8h. Daphnids were fed daily with a 3:1 mixture (based on cell numbers) of the algae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii.* Culture maintenance, medium renewal and exposures were performed in modified M4-medium, as described in Chapter 2 (§2.1).

S7.1.2. European laboratory clones

Seven *D. magna* clones from 7 different European laboratories were investigated. Table S7.1 (Supplementary Material, Table S1) gives a summary of the origin of the different clones used in the present study. Following arrival of subsamples of the monoclonal cultures, they were given a clone ID. Next, an individual of each clone was picked out to established a clonal lineage (Figure S7.1). The maintenance of the laboratory clones was the same as described in chapter 2 (§2.2).

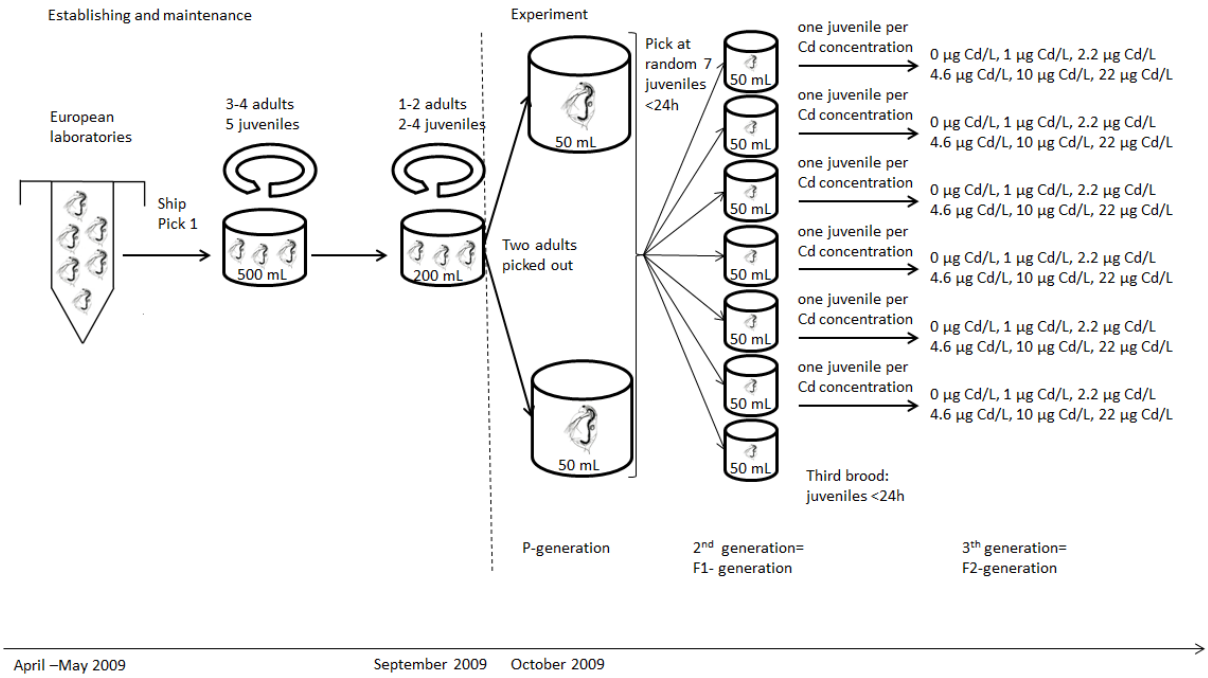

Figure S7.1: Test-design for life-table experiment by European monoclonal *Daphnia magna* populations. This is presented for one clone. Same design was followed for other clone.

S7.1.3. Test design of the exposures to control and Cd

The test design is scheduled in Figure S7.1. In a first step, two adult individuals from each of the 7 laboratory clones were transferred individually to separate 50 mL polyethylene beaker (= P-generation in Figure 7.1). For each clone, juveniles (<24h) produced by these two adults were pooled together and 7 juveniles (<24h) were randomly picked out, to start the second generation (2^{nd} generation, i.e. F1, in Figure 7.1). Each juvenile was transferred individually to a separate 50 mL polyethylene beaker. The individuals in this second generation (F1) then served as the mothers for producing the following generation. At the third brood, six juveniles (<24h) (F2) from six different mother organisms (F1) were selected and were placed individually in 50 mL polyethylene vessels with modified M4 medium and with a Cd range between 1 and 22 µg Cd/L (added as $CdCl_2 \cdot H_2O$) including a control (no added Cd). As such, maternal effects can be ruled out in the estimation of genetic variance, as for each clone in each Cd concentration, each of the six replicate individuals (juveniles)

being exposed originated from a different mother organism. All Cd exposures with all clones were simultaneously initiated, allowing a comparison that is not biased by temporal variability of the cultures. Medium renewal was three times a week and organisms were fed daily with 250 µg dry wt/individual, 500 µg dry wt/individual and 750 µg dry wt/individual in the first, second and third week of their life, respectively. Based on daily observations the following traits were determined: population growth rate (r_m) survival and total reproduction at day 21 (R_0).

S7.1.4. Chemical analyses

Chemical analyses were the same as described in Chapter 2 (§2.4)

S7.1.5. Statistical analyses

Statistical analyses with the laboratory clones were performed with Statistica 6 (Statsoft, Tulsa, OK). The No Observed Effect Concentration (NOEC) values based on net reproductive rate (R_0) were determined with the Mann Whitney U test at the $p<0.05$ significance level. A Bonferroni-Holm correction of p-values was applied following the OECD guideline on the statistical analysis of ecotoxicity data (OECD 2006). In order to estimate the 21d-EC_{10} and 21d-EC_{50}, the following log-logistic models (Eq. S7.1 and Eq. S7.2) were fitted to the concentration-response data, i.e. R_0 as a function of measured dissolved Cd:

$$y = \frac{k}{1+\left(\frac{x}{x_{50}}\right)^{s}} \qquad \text{(Eq. S7.1)}$$

$$y = \frac{k}{1 + \left(\frac{x}{x_{50}}\right)^{\frac{\ln\left(\frac{1}{9}\right)}{\ln\left(\frac{x_{10}}{x_{50}}\right)}}} \qquad \text{(Eq. S7.2)}$$

Where y represents the predicted response (R_0), x is the measured concentration (µg Cd/L), k = the fitted response in the control treatment, i.e. at x = 0 µg Cd/L, s = the slope parameter, x_{50} = the EC_{50} (µg Cd/L), and x_{10} = the EC_{10} (µg Cd/L).

S7.2. Results of 21-day life table experiment with monoclonal *Daphnia magna* laboratory populations

S7.2.1. Physico-chemistry of test media

The physico-chemistry of the test media is presented as supportive information (Table S7.2). DOC ranged between 4.6 and 6.0 mg/L and pH between 7.6 and 7.9. The mean dissolved Cd concentrations (mean of old and new medium) differed at most 17% from the nominal Cd concentration. The Cd concentration in the old medium was on average 21% lower than in the new medium.

S7.2.2. Effect concentration of monoclonal *Daphnia magna* laboratory populations

Considerable variability of reproductive Cd toxicity was observed among the 7 laboratory clones (Table 7.1). The 21d-NOECs of the laboratory clones varied between 0.89 and 8.34 µg Cd/L (9-fold difference, n=4), the 21d-EC_{10}s between 0.31 and 11.4 µg Cd/L (33-fold difference, n=5), and the 21d-EC_{50}s between 3.8 and 20.1 µg Cd/L (5-fold difference, n=6).

Table S7.1: Summarize of the nine different laboratory clones used in this experiment.

Clone ID used in this paper	Origin	Used in following papers	Supplier (Holding in continuous culture)
Clone CZ	Collected from a freshwater reservoir in Brno, Czech Republic	Haeba et al. (2008)	Research Centre for Environmental Chemistry and Ecotoxicology, Masaryk University, Brno, Czech Republic.
Clone K6	Collected from a pond in Kiel, Antwerp, Belgium Cultivated in Ghent University since 1976?	Muyssen et al. (2005)	Laboratory of environmental toxicology, University Gent, Belgium
Clone SE	Collected in a small lake in Bohuslän, cultivated since 1974 in Goteborg University		Department Applied Environmental Sciences, Goteborg University, Sweden
Clone DK	Langedam, Zealand Denmark	Perlt et al. (2009)	Freshwater Biological Laboratory, University of Copenhagen, Denmark
Clone F	North America Testing laboratory	Stuhlbacher et al. (1992) Barata et al. (1998)	IIAB CSIC Environmental Chemistry Department, Barcelona, Spain
Clone A	IRCHA, France Originally from The Water Research Centre, Medmenham UK	Barata et al. (1998) Barata et al. (2000b) Baird et al. (1991). OECD (1997)	Laboratory of Ecotoxicology, CBAS & CIIMAR, University of Porto, Portugal
Clone IRCHA type 5	IRCHA France Originally from The water research Centre, Medmenham UK	Garric et al. (2007)	Cemagref Laboratory of ecotoxicology, Lyon, France

Table S7.2: Physico-chemical characteristics of test media during the Cd exposure experiment. Values represent mean ± standard deviation. NM is new medium. OM is old medium.

Nominal Cd concentration (µg Cd/L)		DOC (mg C/L)	pH	Cd concentration (µg Cd/L)	Mean Cd concentration (µg Cd/L)
0	NM	4.62	7.67	<0.1	<0.1
	OM	5.45 ±0.64	7.58 ± 0.22	<0.1	
1	NM	4.69	7.63	1.06 ± 0.05	0.89 ± 0.22
	OM	5.74 ± 0.34	7.60 ± 0.24	0.73 ± 0.25	
2.2	NM	4.84	7.65	1.96 ± 0.18	1.92 ± 0.06
	OM	5.83 ± 0.52	7.70 ± 0.31	1.88 ± 0.38	
4.6	NM	4.86	7.62	4.56 ± 0.16	3.96 ± 0.88
	OM	5.99 ±0.30	7.73 ± 0.25	3.34 ± 1.37	
10	NM	4.69	7.65	9.79 ± 0.01	8.34 ± 1.99
	OM	5.97 ± 0.67	7.76 ± 0.27	6.94 ± 2.38	
22	NM	4.76	7.66	20.60 ± 0.42	18.87 ± 2.45
	OM	5.89 ± 1.32	7.86 ± 0.27	17.13 ± 1.92	

References to Supplementary Material

Baird, D.J., Barber, I., Bradley, M., Soares, A.M.V.M., Calow, P.. 1991. A comparative study of genotype sensitivity to acute toxic stress using clones of *Daphnia magna* Strauss. Ecotoxicol Environmental Safety, 21: 257-265.

Barata, C., Baird, D.J., Amat, F., Soares, A.M.V.M.. 2000. Comparing population responses to contaminants between laboratory and field: an approach using *Daphnia magna* ephippial egg banks. Functional Ecologyg 14(4): 513-523.

Barata, C., 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(2): 115-137.

Garric, J., Vollat, B., Duis, K., Pery, A., Junker, T., Ramil, M., Fink, G. ,Ternes, T.A.. 2007. Effects of the parasiticide ivermectin on the cladoceran *Daphnia magna* and the green alga *Pseudokirchneriella subcapitata.* Chemosphere, 69: 903-910.

Haeba, M.H., Hilscherova, K., Mazurova, E., Blaha, L.. 2008. Selected endocrine disrupting compounds (vinclozolin, flutamide, ketoconazole and dicofol): Effects on survival, occurrence of males, growth, molting and reproduction of *Daphnia magna.* Environmental Science Pollution Research, 15(3): 222-227.

Muyssen, B.T.A., Bossuyt, B.T.A., Janssen, C.R.. 2005. Inter- and intra-species variation in acute zinc tolerance of field-collected cladoceran populations. Chemosphere, 61(8): 1159-1167.

Perlt, W.T., Mulderij, G., Christoffersen, K.S.. 2009. Clonal variation in physiological responses of *Daphnia magna* to the strobulirin fungicide azoxystrobin. Environmental Toxicology and Chemistry, 28(2): 374-380.

Schafers, C., Boshof, U., Juerling, H., Belanger, S.E., Sanderson, H., Dyer, S.D., Nielsen, A.M., Willing, A., Gamon, K., Kasai, Y., Eadsforth, C.V., Fisk, P.R., Girling, A.E.. 2009. Environmental properties of long-chain alcohols, Part 2: Structure–activity relationship for chronic aquatic toxicity of long-chain alcohols. Ecotoxicology and Environmental Safety, 72(4): 996-1005.

Stuhlbacher, A., Naylor, B.C., Calow, P.. 1992. Induction of cadmium tolerance in two clones of *Daphnia magna* Straus. Comparative Biochemistry and Physiology, 101C (3): 571-577.