Consequences of environmental pollution on genetic diversity in populations of the midge *Chironomus riparius*

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1 Introduction

General introduction

The rate of species extinctions due to anthropogenic activities has dramatically increased within the past few centuries (Dirzo & Raven, 2003; Novacek & Cleland, 2001). Although the mechanisms and ultimate causes leading to the extinction of species remain largely unclear (Frankham et al., 2002), five threats to global biodiversity have frequently been referred to as the most important: habitat destruction and fragmentation, global climate change, hunting and overuse of food resources, biological invasions and environmental pollution (Dudgeon et al., 2006; Lewis, 2006; Novacek & Cleland, 2001). Different research fields, as conservation biology, ecology and ecotoxicology, investigate the effects of these factors on organisms and found strong evidence for their negative impact on regional and global biodiversity.

In most cases, natural populations will be impacted not only by one threat, but rather a combination of them (Buckley & Roughgarden, 2004; Kappelle et al., 1999). Multiple environmental stress factors can have cumulative negative effects on the survival of populations (Sih et al., 2004). To understand, how natural populations respond to combinations of different stress factors is thus of crucial importance in order to understand our present and future impact on all scales of biodiversity (Warren et al., 2001).

The effects of anthropogenically introduced chemicals on organisms and ecosystems are investigated in the field of ecotoxicology. Research in this area has led to a large body of information concerning the impact of chemical stress on the fitness of model species in the laboratory. In contrast to this, there is an obvious lack of knowledge on the effects of contaminants on natural populations and communities (Bickham et al., 2000; Bourdeau et al., 1990). For instance, ecotoxicologists have just started to investigate the impact of environmental pollution on the genetic variability of natural populations (Bickham et al.,

2000; Whitehead et al., 2003). Genetic variation provides the raw material for populations in order to adapt to changing environmental conditions and is thus the substrate for evolution and long-term survival of populations and species (Frankham, 2005). The amount of genetic variation in populations is positively correlated with the effective population size (Frankham, 1996). Habitat destruction and fragmentation has divided the ranges of many species into small and isolated refuges. Without migration from adjacent habitats, isolated populations will decrease in their level of genetic diversity through random loss of alleles (Hedrick, 2000). Frankham (1995) for instance, showed that 32 of the 37 endangered species (which occur in small populations per definition) of different animals and plant taxa display reduced levels of heterozygosity compared to closely related and more frequent species.

In strongly human impacted landscapes, both factors, environmental pollution and habitat destruction, can be expected to occur frequently together. It is thus of crucial importance to investigate the impact of reduced genetic diversity and inbreeding on the response to chemical stress. In addition, chemical exposure has frequently been discussed to have an impact on the extent of genetic variability in exposed populations (Guttman, 1994; Staton et al., 2001; van Straalen & Timmermans, 2002). However, evidence for this 'genetic erosion hypothesis' remained scarce to date, most likely because of the difficulty to single out the impact of pollution stress from a background of multiple factors which influence patterns of genetic variability in natural populations (Belfiore, 2001; Staton et al., 2001; van Straalen & Timmermans, 2002).

Outline of the study

The general scope of this thesis was to investigate if genetic variation decreases in populations exposed to xenobiotics and if reduced genetic variation affects tolerance towards chemical exposure. In detail, we focused on the following questions:

i) Does environmental pollution reduce genetic variability of populations in the laboratory and in the wild?

ii) Are genetically impoverished populations more susceptible towards chemical stress?

iii) Do genetic variation and inbreeding affect the outcome of ecotoxicological exposure tests?

Genetic tools were developed and applied in order to investigate the effects of reduced genetic variation and chemical stress on populations in the laboratory and in the field. The choice of a suitable model species is of crucial importance in ecological genetics and ecotoxicology. The chosen model organisms should ideally be of ecologically important, widely distributed, and easy to culture under laboratory conditions (Lowe et al., 2004). For the experiments conducted in this study, the non-biting midge *Chironomus riparius* Meigen 1831 was chosen as a model organism based on the following reasons. Chiromids are a worldwide distributed family of nematocerans that occupy a wide range of fresh water environments. They dominate many limnic habitats both in biomass and species richness (Armitage et al., 1995). Furthermore, chironomids play a key role in these environments of high detritus consumption rates. In addition, chironomids serve as important food resource for a variety of predators, as fishes, water foul and many invertebrate taxa (Armitage et al., 1995).

The species *Chironomus riparius* is a model organism in aquatic ecotoxicology and is frequently used in sediment and fresh water toxicity tests (Vogt et al., 2007a). The species is easy to culture in the laboratory and has a short generation time of only three to four weeks under laboratory conditions.

In order to measure genetic variation in *C. riparius* populations in the laboratory and the field, microsatellite markers were developed for this species (**Chapter 2**). The markers

were also tested for the closely related species *C. piger* in order to allow for species discrimination of field samples.

The third chapter (**Chapter 3**) addresses the question, whether environmentally concentrations of the highly toxic pesticide tributyltin (TBT) affect genetic variation in *C. riparius* populations. To this end, strains of the species were kept for 12 generations either exposed to TBT or under control conditions and both genetic variation and several life-history traits were monitored over time.

The finding that genetic variation is negatively impacted by chemical stress leads to the question, if genetic erosion, which has been addressed in the previous chapter, influences extinction risk of *C. riparius* populations in polluted environments. In **Chapter 4** a study is described, in which *C. riparius* strains with different levels of inbreeding and reduced genetic variation were exposed to the heavy metal cadmium in different concentrations.

If genetic variation affects susceptibility towards chemical stress, then ecotoxicological exposure tests could be biased due to high levels of genetic impoverishment in caged laboratory cultures. **Chapter 5** documents the extent and rate of genetic impoverishment in ten laboratory strains of *C. riparius*.

The next chapter (**Chapter 6**) addresses the question, if this decreased genetic variation indeed affects laboratory exposure tests. Six genetically characterized test strains from different laboratories were tested for variation in life-history response to cadmium exposure.

All previously described studies were performed under laboratory conditions. **Chapter 7** describes patterns of genetic variation and species composition of *Chironomus* in the highly human-impacted Rhein-Neckar region in Southwest-Germany. This study documents the usefulness of molecular genetic tools, like DNA-barcoding and microsatellite analyses for the discrimination of morphologically cryptic invertebrates, like

Chironomus larvae. In addition, the results document the role of population dynamic processes in field investigations on genetic erosion.

In the last chapter (**Chapter 8**) the results of all previously described studies are shortly summarized in order to present a general discussion on the consequences of environmental pollution on genetic diversity in laboratory and natural populations of *Chironomus riparius*.

2 Development and localization of microsatellite markers for the sibling species *Chironomus riparius* and *Chironomus piger*

Abstract

Five variable microsatellite loci are reported for the non-biting midge species Chironomus *riparius and* Chironomus piger. *All loci show considerable intraspecific variation and species-specific alleles, which allow to discriminate among the two closely related species and their interspecific hybrids, and to estimate genetic diversity within and between populations. Additionally, the loci were localized on* C. riparius *polytene chromosomes to verify their single copy status and investigate possible chromosomal linkage. The described markers are used in different studies with regard to population and ecological genetics and evolutionary ecotoxicology of* Chironomus.

Introduction

Chironomids are a worldwide distributed family of nematocerans which occupy nearly all kinds of fresh water environments (Armitage et al., 1995). They play a key role in many lake and river systems because of their high abundance and their detritus consumption, and they represent an important food resource for many bird and fish species (Armitage et al., 1995). Although several studies investigated population dynamics within the Chironomidae, no suitable DNA based population genetic markers for species of this family have been developed so far. One of the most widely distributed and frequent species, *Chironomus riparius* Meigen is used as a model organism in ecotoxicological sediment biotests (OECD 2004). In order to answer the question if anthropogenic pollution stress has consequences on the genetic diversity of *Chironomus* populations in the laboratory and the field, we developed five microsatellite markers for this species. These markers were tested for their applicability to its sister taxon *Chironomus piger* Strenzke,

which is morphologically highly similar to *C. riparius* and has been shown to form interspecific hybrids with the latter species (Hägele, 1999). Furthermore, the loci were located on the salivary gland chromosomes of the species via in situ hybridization in order to test for single copy status and physical independence.

Methods

For the construction of a genomic DNA library we extracted DNA from 100 individuals of a *C. riparius* culture sampled in Bochum (Germany). After shearing of the genomic DNA by nebulization, 0.5-1.5 kb sized fragments were separated electrophoretically, electroeluted and purified. The fragments were ligated into pUC 19 vector plasmids cut with *SmaI* and then transformed into DA10B *E. coli* host cells via electroporation. To identify fragments containing typical microsatellite DNA sequence motifs, we picked 2700 insert-containing clones on gridded nitrocellulose filters (Schleicher & Schuell, BA85) for colony filter hybridization (Grunstein & Hogness 1975). Clones were screened for microsatellites using six different radioactive labelled synthetic oligonucleotide probes ($(CA_{15}$, $(GA)_{15}$, $(AAT)_{10}$, $(AAG)_{10}$, $(ATG)_{10}$, $(GATA)_6$). Fifty-five hybridizing clones were picked at random, and their inserts were amplified by PCR using standard forward and reverse vector primers. Amplification products were column-purified (Qiagen), sequenced by dye terminator cycle sequencing using the ABI BigDye Version 3.1 kit (Applied Biosystems) and separated on an ABI 3730 capillary sequencer. For genotyping microsatellite loci, fluorescent labeled primer pairs were designed manually or using FASTPCR (Kalendar 2003) for 27 loci containing microsatellite motifs. After optimization of PCR conditions, reactions were performed in a total volume of 10 µl containing 0.25 mM dNTPs, 2.4 mM $MgCl_2$, 1 x reaction buffer (20 mM Tris-HCl, 50 mM KCl; Invitrogen), 0.2 µM of each primer and 0.5 U *Taq* DNA polymerase (Invitrogen). In total, 24 microsatellite regions were successfully amplified and tested for variability using an

ALF DNA sequencer (Pharmacia). All loci were analysed using at least 12 individuals from *C. riparius* field samples. Only five loci showed variability within or between the investigated populations (Table 2.1). Those loci were then amplified for 24 individuals each of *C. riparius* and *C. piger* sampled at different localities within the Rhein-Neckar region in Germany.

Results and Discussion

The observed number of alleles ranged from three to 10 for *C. riparius* and two to 13 for *C. piger*, with expected heterozygosity values ranging from 0.621 to 0.864 (*C. riparius*) and 0.041 to 0.859 (*C. piger*). All markers showed variation within both species. Significant linkage disequilibrium (Fisher´s exact test, $p < 0.05$) was found for *C. riparius* between the loci MSC2 and MSC5 after Bonferroni correction. A significant heterozygote deficit was observed for three loci within *C. riparius* and two loci in the *C. piger* samples (Exact HW test; Guo & Thompson 1992) which is most likely caused by population substructuring. Population genetic parameters were calculated using GENEPOP online version 3.4 (Raymond & Rousset 1995).

To localize the microsatellite loci on the chromosomes, in situ hybridization was performed following published procedures (Hankeln et al. 1988; Schmidt et al. 1988). Briefly, salivary gland polytene chromosomes were prepared from 4th instar larvae of *C. riparius*. Plasmid DNA from microsatellite-containing clones was labelled using biotin-labelled dUTP and nick-translation (Gibco-BRL). Biotinylated probes were hybridized overnight to the denatured chromosomes in 5 x SSC (0.75 M NaCl, 0.075 M Na citrate)/0.5% SDS, and detected after washing in 2 x SSC by rabbit anti-biotin IgG primary antibody and a FITC-conjugated goat anti-rabbit IgG secondary antibody. Positions of hybridization signals were determined on the published chromosomal map of Hägele

(1970). Each of the 5 clones revealed a single hybridization signal, due to presence of unique DNA sequences flanking the microsatellites in the clones (Fig. 2.1).

Table 2.1. Repeat motifs, primer sequences, size range, number of alleles (A), observed (H_O) and expected (H_E) heterozygosity, and GenBank accession numbers of five microsatellite loci for *C. riparius* and *C. piger* (in parenthesis). Population genetic parameters are based on 24 individuals per species sampled in the Rhein-Neckar region (Germany). * indicates significant ($p < 0.05$) heterozygote deficiencies.

locus	motif	primer sequence	size [bp]	A	H_O/H_E	access. no.
MSC1	CA_9	for: CAT CAT CCT TAA CAA CCC AC rev: CTA GCT TTG CAG GCG AGT GC	95-103 (96-100)	8 (4)	0.500/0.860* (0.291/0.328)	DQ408105
MSC2	$(TAA)_9, T_{10}$	for: AGA CTA ATG ACC AGA CTT GC rev: CTT GTG ATG CGA AAA GCC TG	114-141 (112-141)	8 (10)	0.750/0.864 (0.333/0.780*)	DQ408106
MSC3	$(GT)_{14,} T_9, T_6$	for: ACT ACG CGT GCC TCA ACA GC rev: AGC TAA TTC TCA TGT TGG TC	168-176 (159-189)	7 (13)	0.583/0.758* (0.541/0.857*)	DQ408107
MSC4	$(TCA)_6$	for: TGA CTG AAC TTC CGC AAT GGG rev: CCG AGA ATG CTG CGA TCC AG	211-216 (215-216)	3 (2)	0.458/0.621 (0.041/0.041)	DQ408108
MSC5	$(CA)_{11}, A_9$	for: AAC ATT TGA ACG CAC ATC G rev: ATT TGA TTG TAT GTC CTG	264-278 (269-290)	10 (13)	0.750/0.830* (0.666/0.859*)	DQ408109

The in situ hybridization thus verified the 'single copy' status of the microsatellite loci. Possibly, high recombination rates due to the presence of the internally repetitive Balbiani Ring genes on chromosome 4 obliterate linkage of MSC2 and MSC4 alleles. Two loci reside on the small telocentric chromosome IV which contains the nucleolus (MSC2 and MSC4). However, genetic data did not reveal linkage for these loci. The other three loci reside on the large metacentric chromosomes I (MSC1) and III (MSC3 and MSC5). Thus linkage disequilibrium found between the loci MSC2 and MSC5 is not due to close physical location on the same chromosome.

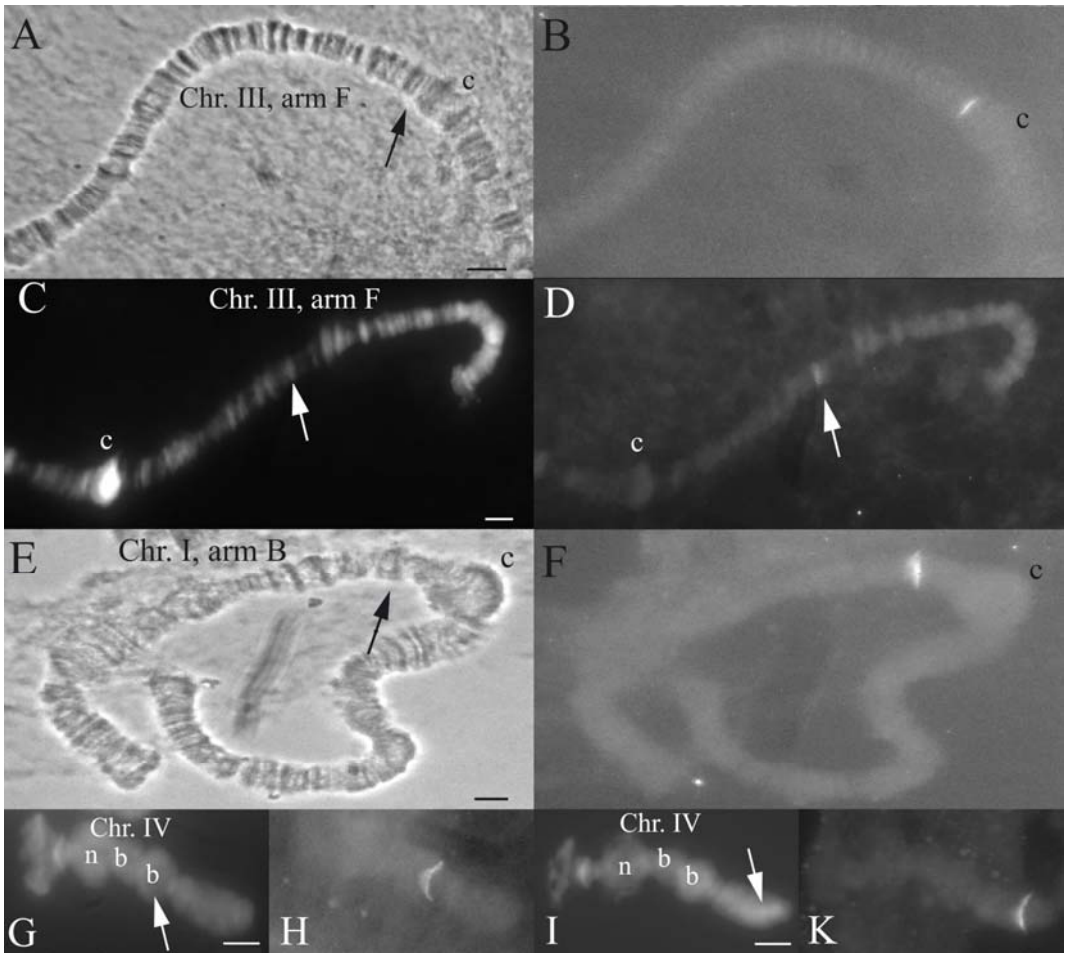

Figure 2.1. Localization of microsatellite loci by in situ hybridization to salivary gland polytene chromosomes of *C. riparius* (MSC5, A and B; MSC3, C and D; MSC1, E and F; MSC2, G and H; MSC4, I and K). Micrographs B, D, F, H and K show the signals of hybridized DNA probes, A and E represent phase contrast pictures of the respective chromosomes and C, G and I show chromosomal banding patterns after staining with the DNA dye DAPI. Arrows indicate positions of hybridization signals. Centromeres (c), nucleoli (n) and Balbiani Ring puffs (b) are indicated. Bars represent 10 µm.

The presented markers are useful tools for the assessment of the genetic population structure of *C. riparius* and *C. piger*. They can be applied for species discrimination due to the occurrence of species-specific alleles at all five loci (data not shown) as well as for the investigation of differentiation and hybridization between both species. The markers are currently used in several projects including studies on the regional population structure of

C. riparius and *C. piger* and the genetic consequences of pollution stress for *C. riparius* populations.

3 Genetic impoverishment in tributyltin exposed strains of the midge *Chironomus riparius*

Abstract

The question if exposure to environmental contaminants can reduce genetic diversity of populations remained unanswered until present. Field surveys failed to provide clear evidence for pollution-induced genetic impoverishment because of the difficulty to extrapolate the influence of a single factor on the genetic structure of populations in natural environments. Thus, we chose a laboratory approach in order to test, if tributyltin exposure leads to reduced levels of genetic variation in the ecotoxicological model organism Chironomus riparius. *Two TBT-exposed and two unexposed strains were reared in the laboratory and life-history traits and levels of genetic variation were monitored for 12 generations.*

Genetic variation significantly decreased in both TBT-exposed strains, while no effects where observed in the controls. TBT had negative effects on all investigated life-history traits. In contrast to the genetic parameters, life-history traits showed no significant alterations over time. Furthermore, we observed high variation in life-history response towards the stressor between strains and generations. Our findings show that chemical pollution even in relatively low concentrations may reduce genetic variation of exposed populations within only a few generations. This loss of intraspecific genetic diversity will be greatest in rare and isolated populations and thus increases the long-term extinction risk of endangered populations in contaminated and stressful environments.

Introduction

Numerous studies have documented negative effects of environmental pollutants on life-history traits of ecotoxicological model species in the laboratory (Walker et al., 2001). Although laboratory test systems provide a powerful tool for toxicity assessment, they mostly consider acute or single-generation effects on life-history response towards chemical exposure (Vogt et al., 2007b). In contrast to single generation experiments, natural populations are chronically exposed to pollutants over multiple generations and numerous factors influence the long-term reaction towards chemical exposure in the field. For instance, populations may adapt to polluted environments (Gillis et al., 2002; Vogt et al., 2007b). Furthermore, chemical exposure has frequently been predicted to reduce genetic variation in natural populations (Bickham et al., 2000; Guttman, 1994; Staton et al., 2001; van Straalen & Timmermans, 2002; Whitehead et al., 2003). This effect, named genetic erosion, has been reported in field surveys on different animal taxa, like fishes (Kopp et all., 1992; Murdoch and Hebert, 1994), crustaceans (Krane et al., 1999; Street et al., 1998 and marine gastropods (Kim et al., 2003). However, most studies in this field show little evidence for erosion of genetic variation due to pollution (e.g. Baker et al., 2001; Brown et al., 2007; Chen et al., 2003; De Wolf et al., 2004; Larno et al., 2001; Muller et al., 2004; Nadig et al., 1998; Prus-Glowacki et al., 1999, Woodward et al., 1996). Thus, the question if genetic erosion threatens populations in polluted habitats remains unanswered (van Straalen & Timmermans, 2002). Patterns of genetic variation within and between natural populations are influenced by various historical and recent factors, like past and present selection processes, migration and chance events (Avise, 2004). Most field studies with regard on the effects of pollution on genetic variation, however, do not consider other processes which affect genetic variation in natural populations (Belfiore & Anderson, 1998; Bickham et al., 2000; Whitehead et al., 2003). We thus chose an experimental approach in order to investigate, if pollutants in environmentally relevant

concentrations reduce genetic variation in populations of an ecotoxicological model organism, the midge *Chironomus riparius* Meigen (Diptera: Chironomidae). *C. riparius* is widely distributed in small streams, ditches, ponds and puddles throughout the holarctic. In organically polluted and muddy habitats, the species may reach high densities and it plays a key role in many freshwater ecosystems due to its high biomass and its activity as a sediment dweller. Furthermore, the species is an important food resource for predatory fish and insect species (Armitage et al., 1995). As detritus feeders living in the upper sediment layers, chironomids get into intensive contact to sediment associated toxicants, like heavy-metals, PCBs or organotin components (Vogt et al., 2007a).

In this study, *C. riparius* larvae were exposed to tributyltin (TBT) contaminated sediment. TBT is an effective pesticide that has been shown to have severe toxic effects on a wide range of aquatic organisms, including chironomids in the laboratory (Vogt et al., 2007a). Furthermore, it has been shown that TBT is responsible for shifts in species composition of aquatic ecosystems (Sayer et al., 2006).

We used a nominal TBT concentration that is environmentally relevant (160 µg Sn/kg sediment dw) and has been previously shown to affect life-history traits of *C. riparius* (Vogt et al., 2007a). The exposure was maintained for 12 generations and genetic variation as well as life-history traits were monitored over time.

In particular, we focused on two main questions:

(i) Does TBT exposure in an environmentally relevant concentration cause a measurable reduction of genetic variation at neutral loci within only a few generations?

(ii) Is it possible to observe changes in life-history response to TBT exposure over time?

In order to determine levels of genetic variability in particular generations, we measured allelic variation at five neutral microsatellite loci. Reduced genetic variation will decrease in small population sizes due to drift effects even in the absence of stress factors

(Frankham et al., 2002). To examine TBT effects on genetic variation, we included two independent control strains that ran parallel with the TBT treatments.

Our findings show that chronic TBT exposure leads to a clear and significant decrease in genetic variability compared in exposed strains, while no evidence for adaptation processes could be observed. We discuss these findings in regard to their general relevance for ecotoxicology as well as for conservation strategies in polluted environments.

Materials and Methods

Experimental procedure and TBT exposure

For the experiments a laboratory strain of *C. riparius* was used which originates from a cross-breeding of 11 different laboratory strains in 2004 (GEN^+; described more in detail in Nowak et al., 2007). The experimental design of the study was as follows: Four strains were reared in the laboratory for twelve generations. Two of the strains were kept under control condition (solvent control = SC), while larvae of the two remaining strains where chronically exposed to sediment-bound tributyltin as described below. To start the experiment, 42 egg masses where taken from the source strain and hatched larvae where carefully mixed in a petri dish. 350 larvae were randomly chosen as start generation for each treatment. Larvae were brought up in seven 2 l glass vessels per treatment containing 1 l reconstituted water and 100 g artificial sediment (pure quartz sand; see Vogt et al., 2007a). Fifty larvae were dispersed into each replicate. For the stressed treatments, the sediment was spiked with 160 µg as Sn/kg sediment dw (390 µg as TBT/kg sediment dw) using pure ethanol as solvent. After larval development, emerged imagines were brought into 10 l reproduction containers (30 x 20 x 20 cm) containing a square water filled box (11.5 x 11.5 x 5.5 cm, 400 ml) for egg laying. The produced egg masses were extracted from the reproduction container and sampled in 24 micro-well plates for hatching. After hatching, 350 L1 larvae were taken from the two days with the highest egg mass

production and mixed in a vessel. Subsequently larvae were randomly distributed on the replicates to start the next generation. The following life history parameters were measured in each generation: larval mortality, mean emergence time (EmT_{50}), produced egg masses per female and hatchability of egg masses. Furthermore, daily population growth rate (PGR; calculated according to Vogt et al., 2007a) was used as an additional endpoint in the experiments.

For the genetic analyses, 200 larvae from the same clutches as those used for the multi-generation study were brought up in each generation in two separate 10 l aquaria with spiked and control sediments. L4 larvae were frozen in liquid nitrogen and stored at –80°C for further analyses.

The experiment was conducted at 20°C (± 0.5°C) under an 8:16 h dark:light cycle following the OECD guideline 218 for *Chironomus* sediment tests (OECD, 2004). For further details regarding the experimental procedure, see Vogt et al. (2007b). The experiments were stopped after the 12^{th} generation.

Genetic analyses

Genetic diversity in the populations was measured using five variable microsatellite markers described in Nowak et al. (2006). Frozen L4 larvae were homogenized in 1.5 ml Eppendorf® tubes containing 700 µl standard CTAB buffer and 4 µl 20 mM proteinase K. Tissue was digested in a water bath at 63°C for 1 h followed by standard chloroform/isoamylalcohol 24:1 treatment. DNA was precipitated in 1 ml isopropanol 100% for 1 h at -20°C and washed twice with 300 ml ethanol 70%. DNA was diluted in 600 µl VE water and stored for further treatment at 4°C. Polymerase chain reaction was performed on a Tetrad thermocycler (Invitrogen®) with cycling conditions as follows: 1 min 92°C, 1 min 55°C, 1 min 72°C, repeated 36 times. Reactions were performed in a total volume of 10 µl containing 0.1 µl of 25 mM dNTPs, 0.5 µl 50 mM $MgCl_2$, 1 µl 10x

reaction buffer, 0.2 µl of each primer (10 pmol) and 0.1 µl 5 U Taq DNA polymerase (Invitrogen®). PCR products were loaded on a 1.4 % agarose gel at 120 V and quantified after staining with ethidium bromide under UV light.

For the fragment analysis, 1 µl DNA solution containing ~1 ng of each microsatellite fragment was mixed with 5 µl dextran blue solution and 1 µl of internal size marker lambda DNA. The solution was loaded on a 9% polyacrylamid gel using an ALF 1 sequencer (Pharmacia®) The gels were run for 450 min at 1000 V. Length of the amplified DNA fragments was calculated using the ALFWIN 3.1 software followed by manual corrections.

Acute tests

In order to detect alterations in TBT susceptibility of *C. riparius* larvae, acute tests were performed with L1 larvae of all four strains in generations 4, 8 and 12. Larvae were exposed to nominal TBT concentrations of 2, 6.3, 20, 63 and 200 µg as Sn/l via water, including a solvent control (10 µl EtOH/l, equals 0.17 µM). After 24 h, the survival rate was determined using a stereo microscope

Statistical analysis

Prior to statistical analysis, all endpoints were tested for normal distribution with the Kolmogorov-Smirnov normality test. Nested two-way ANOVA with repeated measurements was used in order to reveal the influence of TBT on variation in larval mortality and mean emergence time (EmT_{50}) and to test for significant differences in life-history response between strains and generations. We chose an ANOVA design in which strains (SC I, SC II, TBT I, TBT II) were nested into treatments (SC and TBT). The analyses were performed using STATISTICA 7.1. (StatSoft Inc., USA). We tested for the presence of significant linear trends in all life-history traits (mortality, EmT_{50}, produced fertile egg ropes per female, population growth rate) and population genetic parameters

(see below) with the Mann-Kendall test and calculated Sen's slope estimate with the Excel-application MAKESENS 1.0, provided by the Finnish Meteorological Institute. Population genetic parameters (expected and observed heterozygosity [H_E and H_O], number of alleles per locus [N_A], number of polymorphic loci [N_P] and test for deviations from Hardy-Weinberg equilibrium) based on the microsatellite data were calculated using GenAlEx 6 software (Peakall and Smouse, 2006).

Results

Life-history traits

In the multi-generation study TBT had a significant effect on both larval mortality and mean emergence time (EmT_{50}; two way ANOVA, both $p < 0.001$; Table 3.1). Mortalities ranged from 14.2% (TBT II in generation 12) to 100% (TBT I and II in generation 10) in the TBT treatments and from 6.3% (SC I in generation 1) to 80.3% (SC I in generation 6) in the controls; Fig. 3.1 A. Over all generations, mean mortality was higher in the TBT treated strains than in the control strains (43.8% in the treatments compared to 27.8% in the controls). However, only in four generations both TBT treated strains showed higher mortalities than the two respective controls (generations 1, 5, 9 and 10). Because of the extinction of both TBT exposed strains in generation 10, the study was maintained with backup strains that had been isolated from both TBT-exposed strains in the previous generation and were maintained under control conditions in generation 10.

EmT_{50} values where higher in both TBT strains compared to the controls in all generations except of generations 4 and 5 (Fig 1B). We found considerable variation in mortality and mean emergence time between strains and generations. In addition, a significant interaction between treatments and generations as well as between strains and generations was detected (all $p < 0.001$; nested two-way ANOVA, Table 3.1).

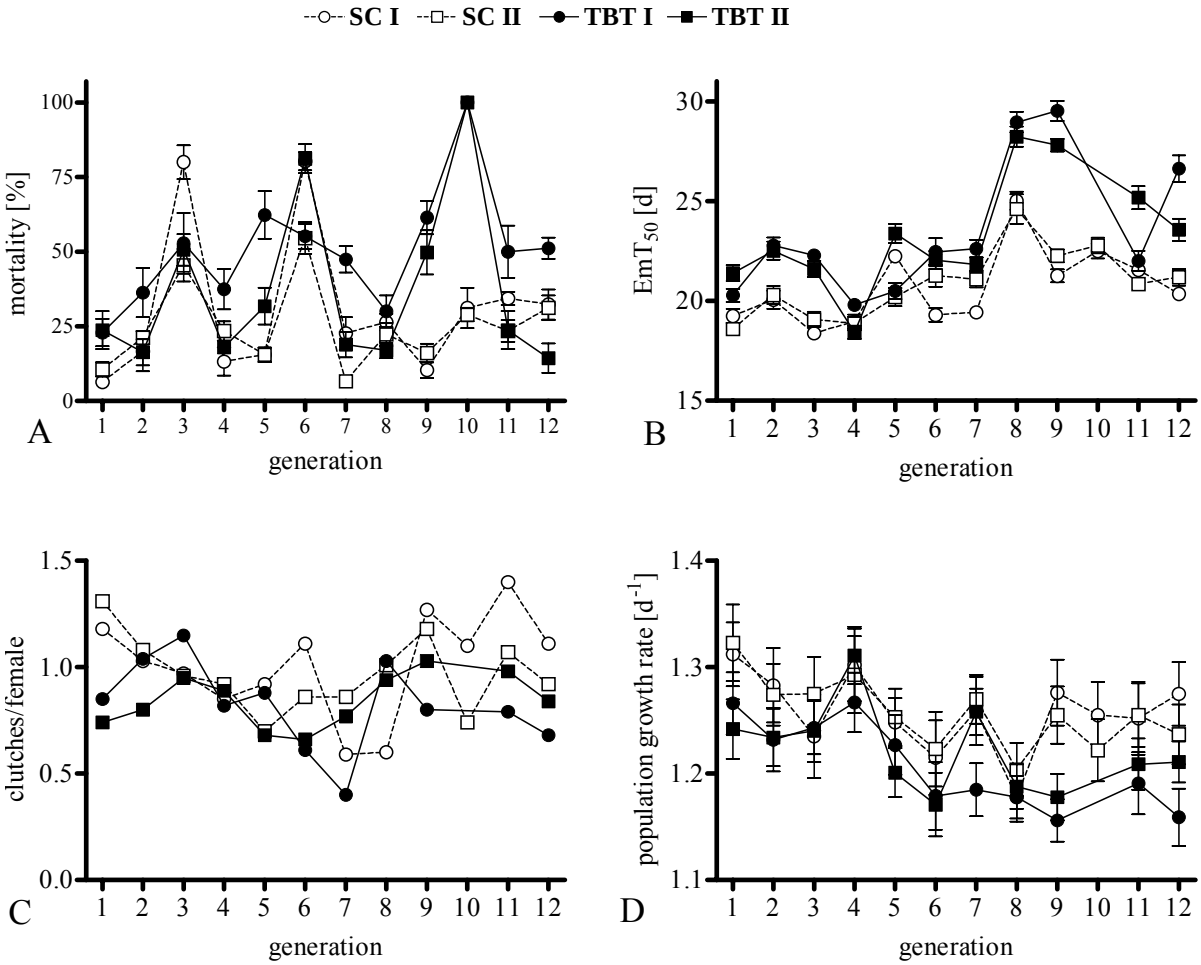

Figure 3.1. Life-history of two TBT exposed (TBT) and two unexposed (SC) *Chironomus riparius* populations over 12 generations. Shown are mean mortality (in %, ± SEM; A), Mean emergence time ($EmT_{50,}$ in d, ± SEM; B), mean number of fertile egg clutches produced per emerged female (clutches/female; C) and the population growth rate (in d^{-1}, ± SD; D).

Number of fertile clutches produced per female varied between 0.40 (TBT I, generation 7) and 1.15 (TBT I, generation 3) in the TBT strains and 0.59 (SC I, generation 7) and 1.4 (SC I, generation 11) in the controls. Over all generations, TBT exposed females produced less egg masses than unexposed females (TBT = 0.83 compared to SC = 0.99).

Table 3.1. Influence of the factors treatment, strain, generation and interaction between these factors on mortality and mean emergence time (EmT_{50}) of *Chironomus riparius*. Shown are degrees of freedom (df), sums of squares (SS), F-values (F) and significance levels (p).

trait	cause	df	SS	F	p
	treatment	1	9482.7	71.960	<0.001
	strain (treatment)	2	9803.1	37.196	<0.001
mortality	generation	10	69185.4	37.719	<0.001
	treatment x generation	10	16375.4	8.928	<0.001
	strain x generation	20	18183.5	4.957	<0.001
	treatment	1	536.8	529.9	<0.001
	strain (treatment)	2	21.1	10.4	<0.001
EmT_{50}	generation	10	1318.4	96.1	<0.001
	treatment x generation	10	19.1	13.9	<0.001
	strain x generation	20	8.2	6.0	<0.001

TBT treated strains showed reduced population growth rates compared to the respective control strains in all but two generations (2 and 4; Fig. 3.1 D) Mean PGR over all generations were 1.22 for TBT (without generation 10) and 1.26 in the controls. No strain showed a significant time trend in larval mortality or fertility (Table 3.2). EmT_{50} values showed a clear tendency to increase over time. However, a significant time trend was revealed only for SC II ($p < 0.05$). Population growth rates tended to decrease with increasing generations in all four strains. Significant effects were observed for SC II ($p < 0.05$) and TBT I ($p < 0.01$).

Genetic variation

All loci exhibited allelic variation in the source strain prior to the start of the experiment (generation 0, data not shown). Allelic richness slightly decreased in the unexposed strains over time. However, a significant negative trend was only found for the expected

heterozygosity in SC II strain (Table 3.2). For all genetic parameters measured, decrease over time was found to be higher in the presence of TBT (Fig. 3.2, Table 3.1).

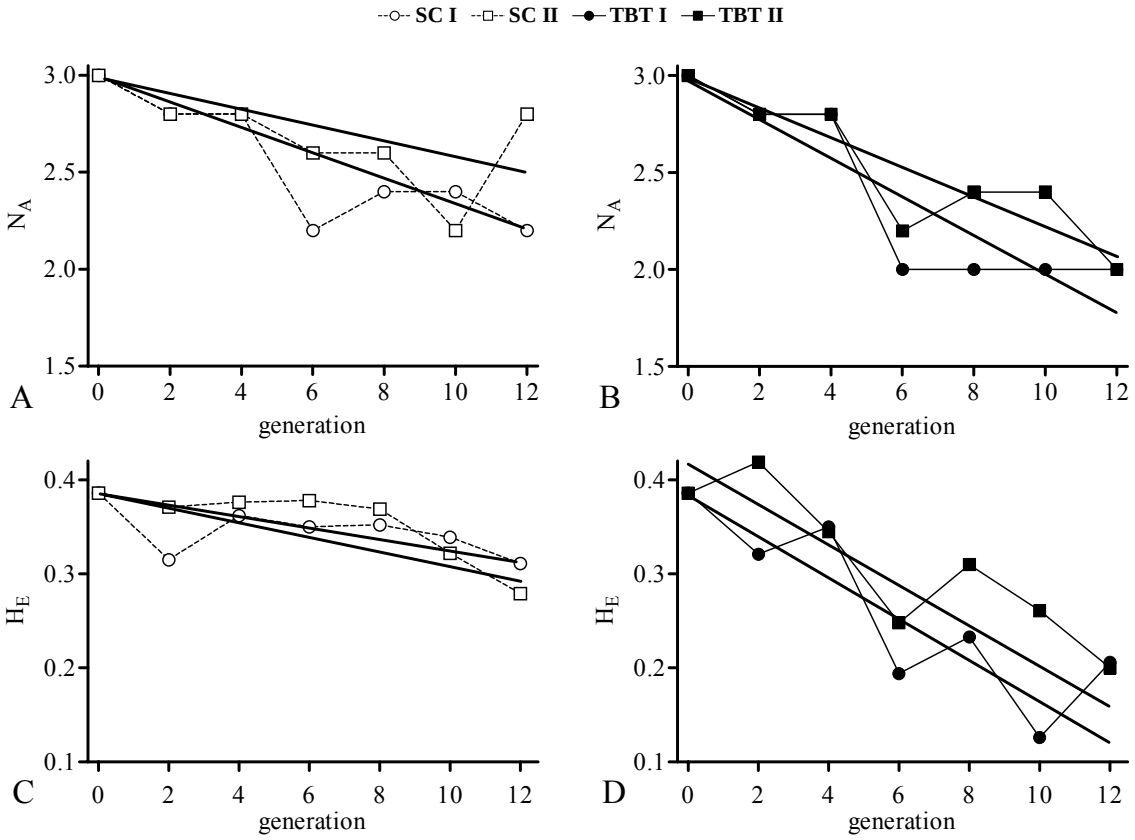

Figure 3.2. Genetic variation (A, B = mean number of alleles per locus [N_A], C, D = expected heterozygosity [H_E]) of two TBT exposed (TBT) and two unexposed (SC) *Chironomus riparius* populations over 12 generations. Lines show slope estimates (see Table 3.2 for values and significance levels).

Number of alleles decreased from 3.0 in the beginning of the experiment to 2.0 after 12 generations in both stressed treatments. Observed heterozygosity (H_O) decreased significantly in both TBT exposed strains (Table 3.2), while no significant trend was observed in the controls. Over time, expected heterozygosity (H_E) decreased significantly only in one exposed strain. However, rates of genetic impoverishment were similar in both TBT affected strains and H_E values decreased from 0.39 in the beginning to 0.21 (TBT I) and 0.20 (TBT II) in the last generation. In contrast, decrease of heterozygosity was far

slower in the absence of sediment contamination (H_E = 0.31 for LMK I and 0.28 for LMK II in generation 12). Under TBT exposure, all genetic parameters showed a significant ($p<0.05$) decrease or at least a trend ($p<0.1$) for decreasing levels of genetic variation at the microsatellite loci (Table 3.2). At the end of the experiments, only one unexposed strain lost variation at one locus. In the presence of TBT both strains lost variation at three of the five loci (data not shown).

Table 3.2. Time series analyses of four genetic parameters and five life-history traits of *C. riparius*. * = $p<0.05$; ** = $p<0.01$; + = $p<0.1$. Ho = observed heterozygosity; H_E = expected heterozygosity, N_A = mean number of alleles per locus; N_P = number of polymorphic loci; EmT_{50} = mean emergence time.

	trait	n	repl.	p level	slope	p level	slope
				SC		TBT	
genetic variation	H_O	7	I		-0.005	*	-0.025
			II		-0.022	*	-0.028
	H_E	7	I		-0.009	+	-0.036
			II	*	-0.013	*	-0.036
	N_A	7	I	+	-0.133	+	-0.200
			II		-0.100	*	-0.150
	N_P	7	I		-0.200	+	-0.500
			II		0.000	+	-0.333
life-history traits	mortality	12	I		2.285		1.946
			II		0.964		0.048
	EmT_{50}	12	I	+	0.183		0.483
			II	*	0.273	+	0.290
	clutches/female	12	I		0.011	+	-0.024
			II		-0.018		0.013
	clutch size	12	I	+	-10.18		-7.825
			II		-6.125		-1.375
	PGR	12	I		-0.003	**	-0.010
			II	*	-0.007		-0.004

Acute tests

LC_{50} values based on the TBT acute tests ranged from 7.33 (SEM ± 1.05, generation 4) to 35.7 (SEM ± 1.01, generation 8) in the strains which were preexposed to TBT (TBT I, TBT II). In the control treatments (SC I, SC II), values ranged from 11.1 (SEM ± 1.05, generation 4) to 25.4 (SEM ± 1.09, generation 12; Table 3.3). No clear differences were observed between preexposed and non-preexposed strains in any test.

Table 3.3. Acute tests with L1 larvae from generations 4, 8, and 12 of the multi-generation study. Shown are calculated LC_{50} values (in µg Sn/l ± SEM).

treatment	strain	gen. 4	gen. 8	gen.12
SC	I	11.1 ± 1.05	21.9 ± 1.04	25.4 ± 1.09
	II	17.2 ± 1.11	21.5 ± 1.04	17.1 ± 1.02
TBT	I	7.33 ± 1.05	35.7 ± 1.01	22.4 ± 1.07
	II	20.4 ± 1.08	23.4 ± 1.07	20.7 ± 492

Discussion

The results presented in this study provide clear evidence that chemical exposure can lead to genetic impoverishment in populations. Although significant stressor effects were observed throughout the study, the impact of TBT on larval mortality, mean emergence time and reproduction was rather low throughout this investigation. However, both the number of alleles and the levels of observed and expected heterozygosity decreased over time in both TBT exposed *C. riparius* strains. The unexposed strains showed just a minor decrease of genetic variation, and no significant negative trends were observed for most genetic parameters.

Chemical exposure may lead to decreased genetic variation of animal and plant populations in different ways. First, selection against less stress-tolerant genotypes may increase frequencies of alleles (potentially leading to fixation) that lead to fitness

advantages under stressed conditions (Bickham et al., 2000). This kind of selection-based reduction of genetic variation, however, will mostly affect loci which are under direct selection or at least physically linked to these. Neutral genetic variation, which can be visualized by microsatellite markers, is not directly affected by selection. However, contaminant exposure may lead to increased mortality and reduced fertility and thus reduce the number of individuals that contribute to the next generation (effective population size, N_e). The amount of allelic variation existing in a population is positively correlated with its effective population size because random genetic drift is more effective in small populations (Frankham, 1996). Thus, chemical stress can reduce neutral genetic variation due to non-selective reduction of N_e.

In this study we obtained no evidence for selection processes, as no significant time trend in any life-history trait was observed and L1 larval tolerance towards TBT did not significantly change over time. However, TBT led to increased mortality and a reduced number of fertile clutches in most generations. Thus, a lower number of individuals contributed to the next generation. Our observation of reduced genetic diversity is therefore due to reduced effective population sizes in the exposed strains, although absolute population sizes were held constant in the beginning of each generation.

This laboratory study was performed without any gene flow between the strains. However, gene flow among populations is very effective in restoring genetic variation (Slatkin, 1987). We thus simulated a small and completely isolated population without migration from adjacent habitats. However, loss of genetic variability is not a problem of widespread species. Rare and endangered species occur per definition in small population sizes and are often reproductively isolated (Amos & Balmford, 2001). Chemical pollution decreases genetic variation in small and isolated populations, which are the main targets of conservation efforts. Hence, our findings provide a functional link between ecotoxicology and conservation genetics.

Besides the investigation of genetic variation under TBT stress, high variation in life-history traits was observed not only between treatments, but also between strains and different generations during the multi-generational study. For instance, no stressor effects were apparent on larval mortality in generations three and eight. In contrast, TBT severely reduced larval survival in other generations (e.g. generations 5, 9 and 10). These findings document the limited significance of single generation surveys in ecotoxicology. Although conditions were kept constant throughout the study and equal concentrations of TBT were applied in all generations, a comparison of different generations (e.g. eight and nine) leads to completely different conclusions concerning the effects of the chosen TBT concentration on *Chironomus* larval survival. There are several ways to explain the large variation in life-history between strains and generations, like heterogeneous contamination of the test sediment or slight variation in test conditions. However, variation in the genetic composition between generations is likely to contribute to the observed life-history variation. For instance, population growth rates of all four strains investigated tended to decrease over time (significant for SC II and TBT I) and decrease was overall slightly stronger in the exposed strains. This decrease can most probably be explained by reduced fitness due to loss of genetic variation in the respective strains. However, 12 generations were considered in this study, which might be not sufficient to detect fitness loss due to reduced genetic variation.

We showed in this study, that toxic substances have the potential to reduce genetic variation within populations before severe population declines due to high mortality rates or low fertility occur. While life-history responses to contamination can be more or less constant over time, resulting in stable population sizes, genetic variation may constantly decrease further over time. The consequences of genetic impoverishment on fitness and tolerance towards chemical exposure and other stressor factors may only be visible over longer time periods. However, loss of genetic variation has to be considered as an

additional threat to populations subjected to chronic environmental pollution (Theodorakis, 2001; van Straalen & Timmermans, 2002).

The increasing use of molecular genetic marker systems, such as microsatellites or AFLP shed new light on the ecological and historical factors which shape patterns of genetic variation (Avise, 2004). However, investigations of genetic variation in chemical exposed populations in the field have to consider various factors that shape the present extent and distribution of intraspecific genetic variation (Belfiore, 2001; Staton et al., 2001; van Straalen & Timmermans, 2002). To extrapolate the influence of pollution on patterns of neutral and selective genetic variation provides an important challenge for both ecotoxicologists and conservation geneticists.

4 Consequences of inbreeding and reduced genetic variation on tolerance to cadmium stress in the midge *Chironomus riparius*

Abstract

Inbreeding and loss of genetic variation are major threats to small and endangered populations. The reduction of fitness due to inbreeding is believed to be more severe under stressful environmental conditions. We generated nine strains of the ecotoxicological model organism Chironomus riparius *of different inbreeding levels in order to test the hypothesis that the degree of genome-wide homozygosity influences the life-history under cadmium exposure. Therefore midge populations were exposed to a gradient of sediment-bound cadmium. The level of genetic variation in the used strains was assessed using microsatellite markers. In the life-cycle tests, inbreeding reduced fitness within* C. riparius *populations both under control and stressed conditions. However, differences between genetically diverse and impoverished strains were greatest at high cadmium exposure. Overall, inbreeding effects were not only dependent on cadmium concentrations in the sediment, but also on the life-history trait investigated. While some parameters where only affected by inbreeding, others were altered by both, inbreeding and cadmium. For the larval developmental time, a significant interaction was found between inbreeding and cadmium stress. While all strains showed a similar developmental time under control conditions, high rates of inbreeding led to a significant delayed emergence time under high cadmium concentrations, resulting in longer generation periods and reduced population growth rates as population-relevant effects.*

The results show, that bioassays with C. riparius *are affected by the level of inbreeding within* Chironomus *test strains. In general, inbreeding is highest in small and isolated populations. Therefore, pollution stress is likely to affect rare and endangered populations more severe than large and genetically diverse ones.*

Introduction

When populations decrease in size, genetic drift can lead to reduced levels of genetic variation. This loss of variation has been shown to enhance extinction risk of populations in the long term due to loss of evolutionary potential which enables populations to adapt to changing environmental conditions (Frankham et al., 2004). Additionally, small populations may suffer from inbreeding due to increased levels of homozygosity of deleterious alleles, which frequently results in the loss of fitness (Charlesworth & Charlesworth, 1987). This process, termed inbreeding depression, has been documented to affect both natural and caged organisms subjected to severe population bottlenecks (Armbruster et al., 2000). In summary, loss of genetic variation and increased genome-wide homozygosity are likely to accelerate extinction processes in plants and animals (Charlesworth & Charlesworth, 1987; Saccheri et al., 1998; Reed et al., 2003).

Furthermore, empirical studies showed that inbreeding depression can be reinforced under stressful environmental conditions, like chemical exposure (summarized in Armbruster & Reed, 2005). For instance, Kristensen et al. (2003) showed that only under environmental stress highly inbred *Drosophila buzzatii* strains produce significantly less progeny than outbred individuals. These findings might have severe implications for both conservation strategies in polluted environments as well as for toxicological exposure assays, for populations of endangered species as well as caged laboratory test strains show reduced levels of genetic variability (Frankham et al., 2004; Joron & Brakefield, 2003; Norris et al., 2001). Furthermore, inbreeding is unavoidable in small populations (Bijlsma et al., 2000). Therefore, it is of high ecotoxicological importance to investigate interactions between inbreeding and chemical stress. These studies will allow to assess the consequences of environmental stress on the fitness of natural populations.

To test if inbreeding and the level of genetic variation influence tolerance to chemical exposure, we generated nine laboratory strains of the non-biting midge *Chironomus*

riparius (Diptera: Chironomidae) which differed in their level of inbreeding (inbreeding coefficient F = 0, 0.125 and 0.375). The level of genetic variation within the strains was verified using microsatellite analysis (Nowak et al., 2006). All strains were exposed to a gradient of sediment-associated Cd and various life-history traits, including reproduction parameters, were recorded.

The main questions of this study were:

(i) Does inbreeding affect life-history traits of *C. riparius* strains in the laboratory?

(ii) Does inbreeding and reduced genetic variation affect susceptibility of *C. riparius* to Cd stress?

(iii) Are 'validity criteria', like thresholds for control mortality, sufficient to prevent test results from bias due to inbreeding effects?

The results of this study are discussed with special emphasis for their consequences on both ecotoxicological test bioassays and species conservation strategies.

Methods

Inbreeding procedure and culture conditions

We established nine independent *Chironomus riparius* strains from a laboratory culture which has been reared in our laboratory for three years. In order to obtain a high level of genetic variation, this culture had been established from a crossbred of 11 laboratory strains from seven different countries (Nowak et al., 2007). The nine strains were generated using different numbers of egg ropes for the founder generation. Three independent highly inbred strains (*high In*) were established performing two generations of full sib mating (inbreeding coefficient F = 0.375). Another three lower inbred strains were obtained by culturing midges in small population sizes crossing the progeny of two egg masses (*low In*, F = 0.125). As a control, three independent replicate strains were kept without generating artificial bottlenecks (*Out*; F = 0).

All strains were equally cultured in breeding cages (60 x 60 x 50 cm) under standard laboratory conditions (temperature 20 ± 1°C, 70% relative humidity and light:dark rhythm of 16:8 h).

Life-cycle experiments

Life-cycle experiments with Cd were performed for each population following OECD guideline 218 (OECD, 2004) with modifications described in Vogt et al. (2007). As test vessels 600 ml glass beakers (Simax, Czech Republic), containing 100 g spiked dry quartz sand as sediment (grain size 0.1 - 0.4 mm, Quick Mix Group, Osnabrück, Germany) and 400 ml reconstituted water (pH-value 7.9 - 8.4; conductivity 540 µS/cm), were utilized. The sediment was spiked with Cd ($3CdSO_4 * 8H_2O$, CAS number 7790843, Merck, Germany; nominal concentrations: 0.3, 0.6, 0.9 and 1.2 mg Cd/kg dw) solved in 300 ml EtOH per kg sediment (Merck, Germany, > 99.5% purity). After spiking, the solvent was evaporated until complete dryness of the sediments. Four replicates were performed per concentration and a solvent control (SC) was included in all nine experiments. In each test vessel 20 first instar larvae were inserted using a stereo microscope (Olympus SZ 40, Hamburg, Germany) and all vessels were covered with gaze (mesh size 0.75 mm) afterwards. Test vessels were gently aerated through Pasteur glass pipettes, which were attached to an air compressor. Larvae were fed daily with 0.5 mg/larva TetraMin® (Tetra Werke, Melle, Germany). Emerged midges of each Cd treatment were collected with an exhauster and transferred into a breeding container (30 x 20 x 20 cm) covered with gaze. A plastic dish (11.5 x 11.5 x 5.5 cm) filled with 400 ml of reconstituted water was provided for oviposition in every container. Egg masses were removed and counted daily. Number of eggs per egg mass was estimated according to Vogt et al. (2007). In order to maintain sufficient swarm sizes, all midges from the three strains of every inbreeding level that emerged in the two highest Cd treatments (0.9 and 1.2 mg Cd/kg dw) were put together into a single breeding container.

Larval mortality, mean emergence time (EmT_{50}), sex ratio, fertile egg masses per female and clutch size were used as endpoints in the experiments. Furthermore an integrated population growth model was derived according to Sibley et al. (1997) and Forbes and Cold (2005). The daily population growth rate (PGR) was used as an integrated endpoint in the life-cycle experiment.

Genetic analyses

In order to measure levels of genetic variation in the *C. riparius* populations used for the experiments, twenty-four 4th instar larvae were analysed using five microsatellite markers (Nowak et al., 2006). DNA was isolated and amplified as described in Nowak et al. (2007). Column-purified microsatellite fragments were run on a CEQ 2000 sequencer (Beckman Coulter, California, USA). Alleles were scored automatically and corrected by eye. The software GenAlEx 6 (Peakall & Smouse, 2006) was used to calculate standard population genetic estimates (observed and expected heterozygosity [H_O and H_E], number of alleles per locus [N_A], Shannon index [I_i], test for deviation from Hardy-Weinberg equilibrium) for each strain.

Residual analysis of sediment and water

After the life-cycle experiments two sediment and water samples were taken from control and each concentration and frozen at -80°C. These samples were analysed for their Cd content at the International Graduated School (IHI) Zittau, Germany according to a DIN EN ISO 17294-2:2005-02 guideline. For the analysis, approximately 100 mg of the respective freeze-dried sediment samples was used. Sediment decomposition was performed using a mixture of 440 µl HNO_3 (65%, subboiled), 200 µl H_2O_2 (30%, suprapur) and 100 µl HF (40%) in a microwave procedure (DIN guideline 13656, MLS 1200 microwave, Lab Systems, Leutkirch, Germany). Subsequently extracts and infiltrated overlaying water were analyzed for Cd using an inductive couplet plasma mass spectroscopy (DIN 38406, Elan 5000, Perkin Elmer, Überlingen, Germany). As reference

materials standard soil GBW 08303 (polluted farmland soil, Bureau of Meteorology, Beijing, China) and TMRAIN reference water (National Water Research Institute, Burlington, Canada) was analyzed to check extraction efficiency, showing recoveries of 97 to 100%. Detection limits were 0.015 µg Cd/l for the water phase and 7.5 µg Cd/kg dw for the sediment.

Statistical analyses

All statistical analyses were performed with the program GraphPad Prism (Version 4.03, San Diego, USA). In order to determine treatment effects (compared to the control), all normally distributed datasets (Kolmogorov-Smirnov test) were tested using one-way ANOVA followed by Dunnett's post test. Non-normally distributed datasets were checked for significant differences using Kruskal-Wallis-test followed by Dunn's post test. Mean emergence time (EmT_{50}) was calculated for each gender as shown in Vogt et al. (2007). Significant differences between treatments and controls were checked using a global fitting procedure according to Motulsky & Christopoulos (2004). All single life-history traits were correlated with the level of expected heterozygosity of the strains using Pearson-correlation. Two-way ANOVA followed by Bonferroni post hoc test was performed to determine the influence of inbreeding and Cd stress on life-history performance and to check for potential interactions between both factors. In the two highest treatments (0.9 and 1.2 mg Cd/kg dw), mean values of the respective inbreeding level were used for all single strains in all statistical analyses.

Results

Residual analysis of sediment and water

At the end of the life-cycle experiments, mean percentages of nominal Cd concentrations in the sediment ranged between 60% (1.2 mg Cd/kg dw) and 74% (0.9 mg Cd/kg dw) of

nominals. Cd concentrations in the water samples varied from 3.87 µg/l (0.6 mg Cd/kg dw) to 9.55 µg/l (0.3 mg Cd/kg dw, Table 4.1).

Table 4.1. Nominal and measured Cd concentrations (± SD) at the end of the life-cycle experiment in the sediment and the water phase (SC = solvent control).

nominal Cd conc. [mg/kg dw]	mean analysed Cd conc. (± SD)		recovery (%, ± SD)
	sediment [mg/kg dw]	water [µg/l]	
0 (SC)	0.03 (± 0.03)	0.04 (± 0.02)	-
0.3	0.19 (± 0.03)	9.55 (± 0.18)	63.3 (± 10.4)
0.6	0.39 (± 0.18)	3.87 (± 2.01)	65.0 (± 30.8)
0.9	0.67 (± 0.21)	5.69 (± 0.33)	74.4 (± 23.2)
1.2	0.72 (± 0.02)	5.89 (± 1.10)	60.0 (± 1.74)

Genetic analyses

Measuring of allelic variation at five microsatellite loci led to observed heterozygosity levels ranging from 0.17 (*high In III*) to 0.41 (*Out III*). Generally, both observed and expected heterozygosity, the number of alleles per locus and the Shannon index were lowest in the highly inbred strains (Table 4.2). H_E values were significantly lowered in the highly inbred strains compared to the outbred populations (H_E = 0.20 in *high In* compared to 0.29 in *Out*, $p < 0.05$, one-way ANOVA with Dunnett's post test).

Life-cycle experiments

Considerable variation of larval mortality was observed both within and between the inbreeding and Cd treatments. No significant differences between the inbreeding levels were observed under control conditions and under low Cd exposure for this trait. In contrast, larval mortality was significantly higher in the highly inbred populations compared to the genetically more diverse strains under high Cd exposure (t-test, Fig. 4.1 A).

Table 4.2. Observed (H_O) and expected (H_E) heterozygosity, number of alleles/locus (N_A) and Shannon index (I_i) of nine C. riparius strains using five microsatellite markers (high In = high inbred strains, low In = low inbred strains, Out = outbred strains). * indicates a significant deviation from Hardy-Weinberg equilibrium at one locus.

strain	H_O	H_E	N_A	I_i
high In I	0.24*	0.23	1.60	0.34
high In II	0.25*	0.23	2.00	0.35
high In III	0.17	0.15	1.40	0.22
Ø (± SD)	0.22 (± 0.04)	0.20 (± 0.04)	1.67 (± 0.31)	0.31 (± 0.07)
low In I	0.35*	0.28	2.00	0.45
low In II	0.29	0.28	1.80	0.42
low In III	0.24	0.24	1.60	0.35
Ø (± SD)	0.29 (± 0.05)	0.27 (± 0.03)	1.80 (± 0.20)	0.41 (± 0.05)
Out I	0.41*	0.31	2.00	0.47
Out II	0.28	0.29	2.00	0.47
Out III	0.28	0.26	1.60	0.37
Ø (± SD)	0.32 (± 0.08)	0.29 (± 0.02)	1.87 (± 0.23)	0.44 (± 0.06)

Due to high mortality in some strains under control conditions, tests with only four of the nine strains met the validity criteria of the OECD guideline ("OECD-valid"; control mortality < 30%, OECD, 2004). No Cd effect was observed in the single "OECD-valid" outbred strain (*Out I*). In contrast, mortality was significantly increased at higher Cd levels (0.6-1.2 mg/kg dw) in the "OECD-valid" highly inbred culture (*high In II*; $p < 0.01$) and in the two "OECD-valid" low inbred populations (*low In I* and *II*; $p < 0.05$; Fig. 4.2).

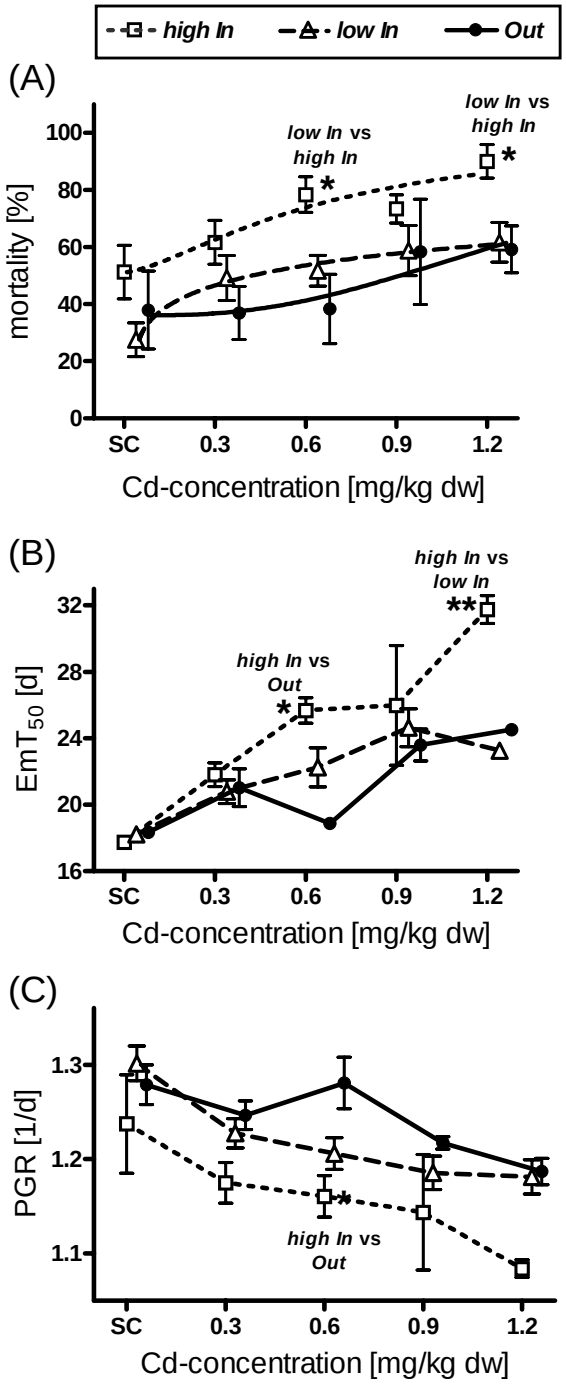

Figure 4.1. Response of differently inbred *Chironomus riparius* strains to cadmium (*high In* = high inbred strains, *low In* = low inbred strains, *Out* = outbred strains, SC = solvent control). **(A):** Mean mortality (%, ± SEM, t-test), **(B):** Mean emergence time (EmT_{50}; ± SEM, Global fitting, **(C):** Mean population growth rate (PGR, ± SEM, two-way ANOVA with Bonferroni post test). * = $p < 0.05$, ** = $p < 0.01$.

Mean emergence time (EmT_{50}) was almost identical in all populations under control conditions (*high In* = 17.7 ± 0.58 d, *low In* = 18.2 ± 0.63 d, *Out* = 18.3 ± 0.48 d). Cd

exposure led to only moderate increase of EmT_{50} values in the outbred and low inbred strains. In contrast, emergence time increased nearly twofold in the *high In* populations at the highest Cd concentration (1.2 mg/kg TG). EmT_{50} values were significantly higher in the *high In* strains than in the outbred populations at two Cd concentrations (0.6 and 1.2 mg/kg TG; Fig. 4.1 B).

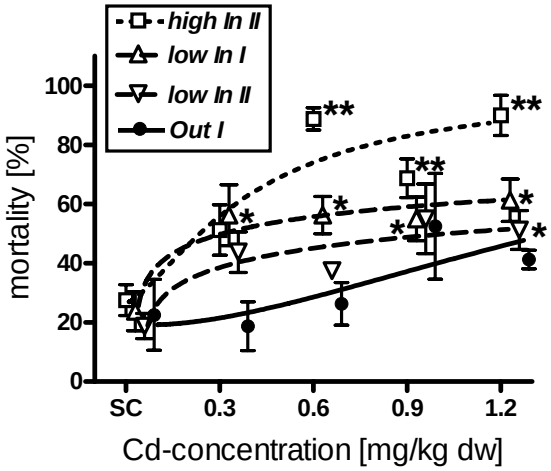

Figure 4.2. Mean mortality (%, ± SEM) of the four *C. riparius* strains with control mortalities below 30% (valid tests) in response to cadmium. Shown are significant differences compared to the respective control (SC). * = p <0.05, ** = p <0.01; one-way ANOVA with Dunnett's post test, n = 4. See Table 4.2 for abbreviations of strains.

Mean proportion of females decreased in the *high In* strains under control conditions significantly (30.7 ± 5.5% compared to 48.7 ± 4.0% in the *low In* and 45.7 ± 9.7% in the *Out* populations). Although the relative proportion of females remained clearly below 50% in all treatments, no significant effects were measured under Cd exposure.

Two-way ANOVA revealed a significant influence of both the inbreeding level and the Cd concentration in the sediment on the larval mortality in the experiment (Table 4.3).

Significant effects of the pollutant and the inbreeding level were also observed for the number of eggs per egg mass and the EmT_{50}. For the latter trait, a significant interaction between the Cd level and the magnitude of inbreeding was found. While the number of

fertile egg masses produced per female was not affected by any variable, female proportions were significantly affected by inbreeding, but not by the exposure level.

For the calculated population growth rate (PGR), highly significant effects were found for both Cd and the level of inbreeding (Table 4.3). PGR were similar under control conditions, with a lowered rate for the highly inbred populations (Fig. 4.1 C). Cd led to a clear decrease of PGR at all diversity levels. However, decline was most severe in the highly inbred populations. Significant differences were observed only between *high In* and *Out* in the 0.6 mg/kg dw treatment (1.16 ± 0.04 d^{-1} to 1.28 ± 0.05 d^{-1}, Fig. 4.1 C).

Table 4.3. Two-way ANOVA for six life-history traits with degrees of freedom (df), F-values and significance levels (p). EmT_{50} = mean emergence time, fertility = fertile egg masses produced per female, PGR = population growth rate.

trait	cause	df	F	p
mortality	Cd	4	5.42	0.002
	diversity	2	10.8	<0.001
	Cd x diversity	8	0.54	0.814
EmT_{50}	Cd	4	21.6	<0.001
	diversity	2	9.43	<0.001
	Cd x diversity	8	2.98	0.014
fertility	Cd	4	0.33	0.853
	diversity	2	1.06	0.358
	Cd x diversity	8	0.74	0.658
clutch size	Cd	4	3.69	0.015
	diversity	2	3.40	0.047
	Cd x diversity	8	2.20	0.056
sex ratio	Cd	4	0.18	0.945
	diversity	2	5.90	0.007
	Cd x diversity	8	1.25	0.304
PGR	Cd	4	8.70	<0.001
	diversity	2	12.7	<0.001
	Cd x diversity	8	0.63	0.743

Besides the direct assessment of the effects of inbreeding on fitness components, associations between expected heterozygosity levels among strains and life-history responses were investigated (Table 4.4). Significant negative correlations between the level

of heterozygosity and larval mortality were found for all treatments. However, significance levels were highest in the upper concentration range (0.9 and 1.2 mg/kg dw). No effects were found for the EmT_{50} and the proportion of fertile egg masses produced per female under control conditions. In contrast, significant correlations were obtained for both parameters under Cd exposure. The proportion of females was affected by the level of genetic variation both under benign and stressed conditions (0.9 mg/kg dw). In contrast to all other comparisons of heterozygosity and life-history traits, no significant correlations were found between the genetic variation among strains and the clutch size in any Cd treatment (Table 4.4). The population growth rate was significantly ($p < 0.01$) correlated to the level of allelic variation in the populations investigated both in the solvent control and three of four Cd treatments. As for the mortality, strongest correlations were found in the two highest Cd treatments.

Table 4.4. Correlations between expected heterozygosity (H_E) and six life-cycle parameters at different Cd concentrations with correlation coefficients and significance levels (EmT_{50} = mean emergence time, PGR = population growth rate, SC = solvent control, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$; Pearson correlation, n = 9).

Cd (mg/kg dw)	mortality	EmT_{50} (♂+♀)	fertility	clutch size	sex ratio	PGR
0 (SC)	-0.74*	0.27	0.60	-0.13	0.79*	0.75*
0.3	-0.74*	0.20	0.83**	0.03	0.60	0.77*
0.6	-0.74*	-0.54	0.13	-0.25	0.32	0.66
0.9	-0.91***	-0.63	-0.76*	0.70*	0.83**	0.84**
1.2	-0.83**	-0.78*	0.81**	-0.76*	0.06	0.81**

Discussion

To our knowledge, we present the first study which investigates the combined effects of inbreeding, reduced heterozygosity and chemical stress with an ecotoxicological model species under standardized conditions (OECD guideline). Recovery rates of Cd in the experiments were similar between the replicates and treatments. Thus, observed variation in life-history response between *Chironomus riparius* strains can not be explained by variations in sediment contamination between the tests. The life-cycle experiments show that life-history traits of *C. riparius* populations were affected both by the amount of Cd in the sediment and the level of inbreeding within the populations and individual genome-wide homozygosity, as verified by the microsatellite analysis. Two-way ANOVA revealed a significant influence on the life-history response in the tests of both the Cd and level of inbreeding (Table 4.3). For most life-history traits, differences between inbreeding levels were higher under exposed conditions (Fig. 4.1 A-C). Furthermore, a significant interaction was found between the severity of Cd exposure and the inbreeding level for the larval developmental time (shown as EmT_{50}, Fig. 4.1 B). Emergence time was extremely delayed in the presence of Cd only in the highly inbred strains, although larval developmental time was not affected by inbreeding in control treatments.

Several studies show that inbreeding depression can be more severe in stressful environments than under benign conditions (Bijlsma et al., 2000; Kristensen et al., 2003). For instance, Armbruster & Reed (2005) show in a meta-analysis of 34 data sets, that inbreeding depression often increases under environmental stress conditions, like heavy metal exposure or temperature stress. However, many studies show just weak correlations between fitness and stress level and ambiguous patterns were observed in some surveys (Cheptou et al., 2000; Dudash, 1990; Miller, 1994).

Different mechanisms can be responsible for the observed differences in stress tolerance. Under chemical stress, complex alterations of gene expression take place that allow for

differential stress response. Inbreeding leads to the expression of previously rare, deleterious alleles. Novel expressed alleles at loci which are involved in stress response could lower the efficiency of complex stress response mechanisms. In addition, heterozygous individuals tend to have lower basic metabolic rates (heterosis effect; Mitton, 1993). Thus, inbred, homozygous individuals have less capacity for energy consuming processes like cellular stress response.

C. riparius is a highly mobile and frequent species which occurs in large population sizes with presumably high gene flow between adjacent habitats. This assumption is confirmed by preliminary results in our laboratory (Nowak, unpublished data). Consequently, reduced genetic variation and strong inbreeding is not expected to occur frequently in natural populations of *C. riparius*. Genetic impoverishment in the laboratory must thus be regarded as an artificial bias for ecotoxicological tests which hampers the transferability of laboratory tests to natural conditions. Although genetic impoverishment might not be of relevance for *C. riparius* in the field, our findings have important implications for species conservation issues. Many endangered species have reduced levels of genetic variation compared to widely distributed and more abundant species (Frankham et al., 2004). Additionally, threatened populations occur in small effective population sizes and are therefore affected by inbreeding. While the role of inbreeding for the extinction of rare populations and species has been debated controversially (Brook et al., 2002), recent studies assign genetic effects like inbreeding to have at least a reinforcing function in extinction processes (Brook et al., 2002; O'Grady et al., 2006). Our study confirms that inbreeding depression is higher under chemical stress. Hence, small and endangered populations will be more susceptible to chemical pollution than large and genetically diverse ones. Already in low concentrations, chemical pollution could thus contribute to local extinction of small and isolated populations which are the main targets for species conservation efforts.

Besides the relevance for conservation biology, our findings have strong implications for ecotoxicological exposure tests. We have shown that inbreeding might not be detected under standard environmental conditions, as the results of the 'OECD-valid' tests illustrate. In these tests, mean control mortalities were below 30% (Fig. 4.2), as requested in the official guideline for *Chironomus* tests. While the outbred strain did not react significantly to the stressor even under higher levels of Cd exposure, the stressed inbred populations showed significantly reduced larval survival. Similar effects were observed for the larval development time and the reproductive fitness (Fig. 4.1 B and C). These results document that threshold levels, like the 30% mortality level for the OECD *Chironomus* test (OECD, 2004) are not sufficient to prevent exposure assays from potential bias due to inbreeding effects. Inbreeding depression can be enhanced under stressful conditions, while no effects are visible in the control treatments. This effect will lead to repeated overestimation of chemical effects on life-history traits when inbred *Chironomus* strains are used in exposure assays.

In order to maintain genetic variation in caged populations of sexual test populations, we recommend regular refreshments with individuals obtained from the field. This artificial gene flow will restore genetic variation and prevent biased test outcomes due to reduced heterozygosity levels.

Additionally, *Chironomus* test strains could be monitored regularly for their allelic diversity using microsatellite analysis (Nowak et al., 2007). Heterozygosity levels in the inbred strains were all lower than those of the outbred strains. Consequently, we observed significant correlations between the levels of heterozygosity at the microsatellite loci and various life-history traits both in control and stressed treatments. Thus, the five microsatellite loci used in this study provide a sufficient discriminatory power in order to monitor genetic variation in *C. riparius* strains effectively.

5 Genetic impoverishment in laboratory cultures of the test organism *Chironomus riparius*

Abstract

Genetic diversity among laboratory and field populations of Chironomus riparius *was investigated using microsatellite DNA analysis. Individuals of midge cultures reared in ten different laboratories showed a clear reduction in the number of alleles per locus and the level of heterozygosity compared to two natural populations sampled in Southern Germany. To reconstruct the rate of genetic impoverishment under laboratory conditions, genetic diversity was monitored in a* C. riparius *laboratory population for 23 generations. Additionally, 11 populations originating from different laboratories were crossed and genetic diversity was compared among `pure´, `crossed´ and natural populations. The results demonstrate that* C. riparius *strains used in standard toxicity tests exhibit only low levels of genetic variability. In addition, our experiments showed that refreshment of stocks with individuals from other laboratories is not sufficient to restore genetic variation. Potential consequences of genetic impoverishment and improvements for toxicological bioassays are discussed.*

Introduction

Life-cycle bioassays represent an important tool in the field of ecotoxicology to assess the toxicity of anthropogenically introduced substances on model organisms in the laboratory (Fent, 2003). However, the transferability of toxicity data obtained from laboratory studies to natural conditions is limited for many reasons. For instance, variation in the bioavailability of chemicals is considerable and various interactions among stress factors may exist in natural environments (Bourdeau et al., 1990). Furthermore, bioassay procedures are not designed to map population dynamic processes which influence the response to chemical exposure (Bickham et al., 2000). In addition, the fitness of organisms

has been shown to be associated with the level of genetic variation within populations (Mitton, 1997). In general, we assume that the long-term survival of populations depends on a sufficient level of genetic variation which enables them to adapt to environmental changes (Frankham, 2005). The loss of genetic variation is associated with inbreeding (Hansson & Westerberg, 2002; Keller & Waller, 2002) which leads to phenotypic expression of previously rare deleterious alleles and thus reduces the fitness within populations also in the short term. Inbreeding depression has often been reported in agricultural stock-breeding as well as in captive breeding programmes for species conservation purposes (Woodworth et al., 2002). While investigations of genetic variation of captive breeding are commonly applied in the latter field (Montgomery et al., 1997), few attempts have been conducted to assess genetic diversity within animal cultures used for toxicological life-cycle bioassays. Inbreeding depression can be more severe under stressful environmental conditions, e.g. salinity, temperature stress or chemical exposure (Armbruster & Reed 2005; Dahlgaard & Hoffmann, 2000; Hauser & Loeschcke 1996; Kristensen et al., 2003). Therefore, high rates of genetic impoverishment in caged test cultures used for exposure assays are likely to influence results of toxicity tests and thus could bias comparability among tests.

In order to assess the level and rate of genetic impoverishment among laboratory cultures used for toxicological bioassays, we measured the level of genetic diversity in ten populations of the non-biting midge *Chironomus riparius* originating from different laboratories across Europe. *Chironomus riparius* is used frequently as a standard test species in sediment toxicity tests and plays a key role in many aquatic ecosystems due to its high abundance, detritus consumption, and importance as a food resource for many fish species, birds, and predatory aquatic insects (Armitage et al., 1995). Genetic variation was compared among 10 laboratory stocks, two natural *C. riparius* populations and one outcrossed population (GEN^+, based on 11 `pure` stock populations). Genetic diversity of

GEN$^+$ population was monitored over 23 generations to document the rate of genetic impoverishment in a caged midge culture. Our main intention was to test if genetic variation is reduced in laboratory stocks of *C. riparius*, and if genetic variation can be effectively restored by refreshing culture stocks with individuals from other laboratories. The results are discussed in relation to their potential consequences for toxicity testing using laboratory cultures.

Methods

Origin of populations

Laboratory strains of *C. riparius* were obtained from twelve laboratories across eight countries in Europe and the United States. The samples were sent as egg ropes or alcohol samples (Table 5.1). Larvae hatched from egg ropes were brought up in 2 l glass vessels filled with 100 g of sterilized quartz sand and 1 l reconstituted water. One hundred larvae from each location were frozen in liquid nitrogen for genetic analysis when reaching L4 larval stage. Eleven laboratory strains were established in our laboratory as permanent cultures. Two *C. riparius* populations were sampled in the field in the Rhein-Neckar Region in Baden-Württemberg/Germany and stored in Ethanol 80% for further analyses.

Crossing procedure and culturing of GEN$^+$ strain

All eleven laboratory strains used for cross-breeding were reared for at least two generations prior to crossing procedures to allow for acclimatisation to new laboratory conditions. For inter-strain crossing, 13 males and 13 females from each laboratory strain were placed into a reproduction cage (60 x 60 x 50 cm). Produced egg masses were transferred into water filled plates for larval hatching. Larvae hatched from fertile egg masses were mixed in a petri dish and 500 individuals were randomly chosen and brought up in ten 2 l glass vessels following the recommendations of the Organisation for Economic Cooperation and Development (OECD) guideline for *Chironomus* tests (OECD, 2004).

Table 5.1. Type, origin and abbreviations of *Chironomus riparius* samples subjected to crossbreeding (cross) and/or genetic analysis (µsat; "+" = included, "-" = not included). "Type of sample" denotes if samples were obtained from a laboratory or directly sampled in the field. "Origin" indicates if the laboratory strains derive originally from a laboratory or a field sample ("-" = unknown origin). Coordinates are provided for the field samples.

abbr.	sample type	location	origin	µsat	cross
AMS	laboratory	University of Amsterdam, The Netherlands	field	+	+
AQS	laboratory	AquaSense BV, Amsterdam, The Netherlands	field	+	+
AST	laboratory	AstraZeneca, Brixham Env. Lab., United Kingdom	-	-	+
CAR	laboratory	Cardiff University, United Kingdom	field	+	+
FFM	laboratory	Goethe-University, Frankfurt, Germany	lab.	+	+
JOU	laboratory	University of Joensuu, Finland	lab.	+	+
LYO	laboratory	Cemagref, Lyon, France	-	+	+
PTG	laboratory	University of Aveiro, Portugal	lab.	+	-
RNS	laboratory	Centre INRA de Rennes, France	lab.	+	+
RIZ	laboratory	RIZA, Lelystad, The Netherlands	field	+	+
SOF	laboratory	Bulgarian Academy of Sciences, Sofia, Bulgaria	field	+	+
OHO	laboratory	Ohio State University, Columbus, United States	field	-	+
GEN^+	laboratory	crossbred of 11 laboratory stocks		+	
KAH	field	ditch near Hanhofen, Germany 49.3159°N 8.3564°E		+	-
SSB	field	ditch near Sulzbach, Germany 49.5813°N 8.6471°E		+	-

The GEN^+ culture was reared under controlled conditions for 23 generations in the laboratory. Population sizes were maintained between 350 and 500 larvae, and seven to 172

fertile egg masses (mean = 61.2 +/- 39.1 standard deviation [SD]) were used to establish the next generation.

Genetic analyses

Genetic variation of 10 laboratory and two field populations was measured at five variable microsatellite loci (Table 5.1). Extraction of DNA was performed using a slightly modified standard cetyltrimethylammonium bromide (CTAB) protocol (Winnepenninckx et al., 1993). Briefly, complete L4 larvae were homogenized and incubated in 700 µl of cetyltrimethylammonium bromide buffer with 57 µg/ml of Proteinase K for at least 45 min at 62°C. After standard chloroform treatment, DNA was precipitated in 1.5 volumes of absolute isopropanol for 1 h at -20°C followed by two ethanol (70%) washing steps and resuspension in water. Microsatellite fragments were amplified using a T3 thermocycler (Biometra, Göttingen, Germany) as described in Nowak et al. 2006. Amplified DNA fragments were diluted 1:25 prior to fragment length analysis (ALF sequencer, Pharmacia Biotech, Uppsala, Sweden) and alleles were scored using the ALFWIN 1.0 software (Pharmacia Biotech, Uppsala, Sweden).

Statistical analyses

Population genetic parameters (observed and expected heterozygosity, number of alleles per locus, Shannon index and Hardy-Weinberg equilibrium) were calculated for each sample using GENEPOP 3.4. (Raymond & Rousset, 1995). Principal Coordinates Analysis (PCA) based on the microsatellite data of 10 laboratory populations and the crossbred population (F2 generation after initial cross) was performed using the program GENALEX 6 (Peakall & Smouse, 2006). One-tailed Mann-Whitney U-test was used to compare the levels of heterozygosity (H_E) between laboratory samples originating from the field (University of Amsterdam, Amsterdam, The Netherlands [AMS]; AquaSense BV, Amsterdam, The Netherlands [AQS]; Cardiff University, Cardiff, United Kingdom [CAR]; Bulgarian Academy of Sciences, Sofia, Bulgaria [SOF]; RIZA, Lelystad, The Netherlands [RIZ]) or

from other laboratory stocks (Goethe-University, Frankfurt a. M., Germany [FFM]; University of Joensuu, Joensuu, Finland [JOU]; Centre INRA de Rennes, Rennes, France [RNS]; University of Aveiro, Aveiro, Portugal [PTG]; see Table 5.1). Two-tailed Mann-Whitney U-test was used to check for significant differences between the level of genetic variation in the first 10 ($n = 7$) and the last 13 ($n = 7$) generations of the Gen^+ strain.

Results

Genetic variation was measured among ten laboratory and two field populations (using 21-25 individuals per population; Table 5.2).

Table 5.2. Population genetic parameters calculated for 10 laboratory and two field samples of *C. riparius*. Shown are sample sizes (*n*), number of alleles per locus (A), proportion of rare alleles with frequencies below 5% (A <5%), observed (H_O) and expected (H_E) heterozygosity and the Shannon index (SI).

pop.[1]	*n*	A	A <5%	H_O	H_E	SI
AMS	23	2.0	0.00	0.469	0.403	0.58
AQS	24	1.8	0.00	0.350	0.332	0.47
CAR	24	2.2	0.00	0.389	0.410	0.65
FFM	25	1.2	0.00	0.040	0.049	0.08
JOU	24	1.8	0.11	0.280	0.307	0.43
LYO	24	1.2	0.00	0.042	0.038	0.07
PTG	24	1.6	0.38	0.123	0.156	0.25
RNS	24	1.6	0.13	0.175	0.176	0.26
RIZ	24	1.6	0.12	0.175	0.204	0.29
SOF	23	2.0	0.20	0.236	0.189	0.31
KAH	24	5.6	0.18	0.625	0.625	1.34
SSB	21	5.0	0.16	0.611	0.596	1.23

[1]See Table 5.1 for definition of abbreviations.

All populations show variability at least at one microsatellite locus; average numbers of alleles per locus range from 1.2 (FFM; Cemagref, Lyon, France [LYO]) to 5.6 in the KAH field sample, while observed heterozygosity ranges from 4% (FFM) to 62.5% (KAH). Both

field samples display variation at all five loci and show comparable levels of microsatellite variation. All laboratory populations have fewer alleles per locus compared to the field populations (on average 1.7 to 5.3 in the field; Table 5.3).

Table 5.3. Average genetic variation of the crossbred population (GEN^+), the laboratory strains (n = 10) and the field samples (n = 2). See Table 5.2 for abbreviations. Mean values of the laboratory populations are shown +/- standard deviation.

abbr.	A	H_O	H_E	SI
GEN^+	3.6	0.47	0.60	1.05
lab.	1.7 +/- 0.33	0.23 +/- 0.14	0.23 +/- 0.13	0.34 +/- 0.19
field	5.3	0.62	0.62	1.28

Half of the laboratory populations show no rare alleles with a frequency below 5% at any locus. Levels of both observed and expected heterozygosity from laboratory cultures are all below the levels measured in the field samples (on average 0.23 in the laboratory compared to 0.62 in the field). A similar pattern was observed for the Shannon index (0.34 in the laboratory compared to 1.28 in the field samples). Additionally, one-tailed Mann-Whitney U-test revealed that within the laboratory stocks, those originally collected from the field have a significantly (p = 0.032) higher level of genetic variation (H_E) than those obtained from other laboratory cultures.

Both the average number of alleles per locus and the Shannon index are higher in the GEN^+ population than in all laboratory strains (Table 5.3). However, genetic variation is still lower than in both natural populations (5.3 alleles per locus among the field populations compared to 3.6 alleles per locus in the cross-breeding).

The two main axes of the Principal Coordinates Analysis explained 63.2% of the total variation and showed that most laboratory strains are differentiated small clusters, whereas

the crossbreed population represents a central and divergent group overlapping with nearly all `purebred´ populations (Fig. 5.1).

Some populations (LYO, FFM) are composed of only two multi-locus genotypes (MLGs). However, the three populations with the highest levels of heterozygosity (CAR, AMS, AQS) appear as differentiated groups with a high proportion of discrete MLGs.

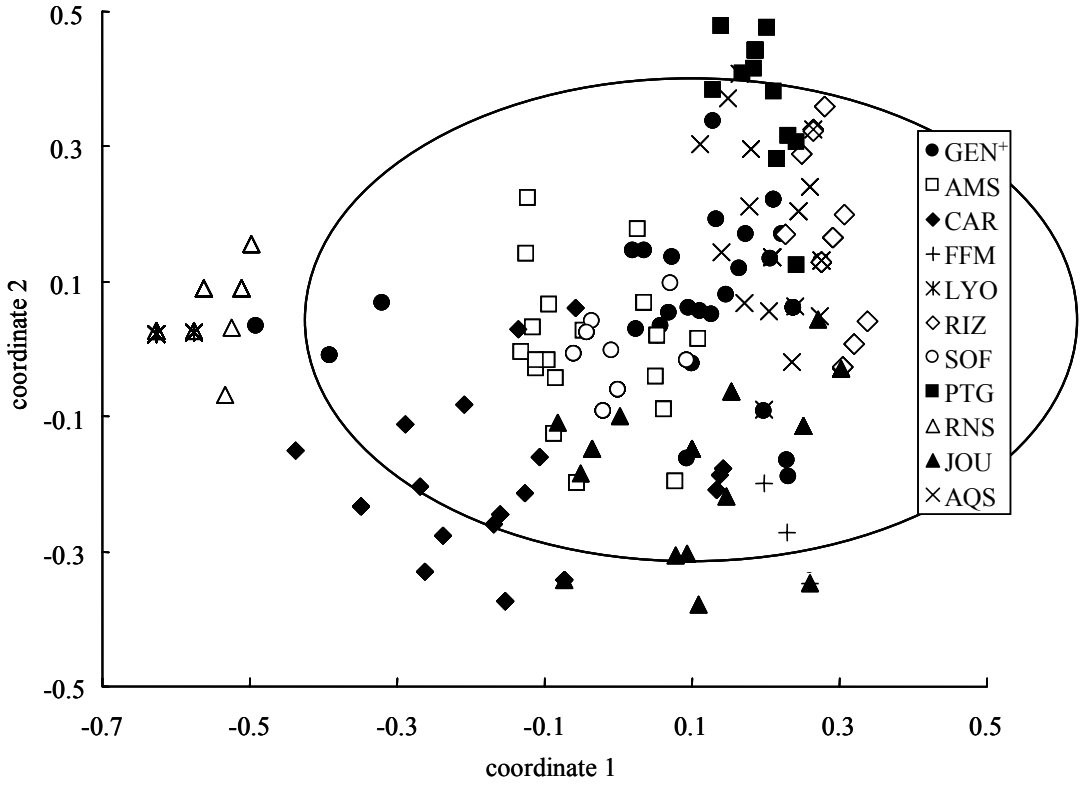

Figure 5.1. Principal Coordinates Analysis (PCA) of 10 laboratory and a crossbred population (GEN^+) of *Chironomus riparius*. Principal coordinate 1 (x axis) and principal coordinate 2 (y axis) explain 42.08% and 21.08% of total variation, respectively. The area within the oval represents the region with the 95% confidence limits for the GEN^+ population. See Table 5.1 for definition of abbreviations used on the figure.

The level of heterozygosity measured in a *C. riparius* strain that was reared in the laboratory for 23 generations (Fig. 5.2) started with H_E = 0.481 in the first generation. Within 15 generations, diversity level dropped to H_E = 0.350 and reached H_E = 0.339 in the last generation. Heterozygosity level in the last 13 generations was significantly below that in

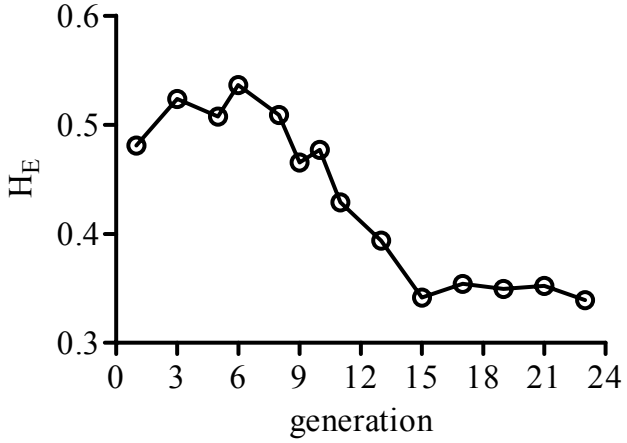

Figure 5.2. Expected heterozygosity (H_E) of a *Chironomus riparius* population (GEN^+) at five microsatellite loci during 23 generations of culturing in the laboratory.

the first 10 generations (Mann-Whitney U-test, p <0.001). The mean number of alleles per locus also decreased from 4.0 in the beginning of the experiment to 2.4 in the last generation surveyed (data not shown).

Discussion

Although the genetic composition of experimental populations is likely to influence the outcome of ecotoxicological bioassays (Duan et al., 1997; Mukhopadhyay at al., 1997; Woods et al., 1989), relatively little attention has been addressed to empirically determine the role of genetic diversity in laboratory test systems. Here we present the first comparison of microsatellite variation between laboratory and natural populations of an ecotoxicological model species. Our results show that laboratory stocks of *C. riparius* are genetically impoverished compared to natural populations (although we are aware of the fact that measuring genetic diversity of two natural populations might not reflect the overall genetic

variation across the whole species range). A similar pattern, i.e. a low degree of genetic variation in laboratory populations has been reported for a wide range of animal taxa, such as other dipteran species (Mukhopadhyay at al., 1997; Norris et al., 2002; Woods et al., 1989), amphipods (Duan et al., 1997), trematods (Stohler et al., 2004) or rodents (Razzoli et al., 2003). For instance, Woods et al. (1989) found a reduced degree of protein variation in laboratory cultures of the closely related species *Chironomus tentans*. Hence, genetic impoverishment may be a commonly occurring phenomenon in caged populations with a limited population size.

Two main mechanisms may explain the reduction in allelic variation in caged populations. Laboratory populations are established by a limited number of individuals from the field or other laboratory strains. This event will result in a "founder effect" caused by establishment of a new population by a small number of founder individuals which carry just a limited proportion of the source populations' initial allelic variation. This stochastic process results in a lowered level of genetic variation in founder populations compared to the source populations, even if they increase in size. Since laboratory populations are reproductively completely isolated, they lack gene flow from variable populations to counterbalance genetic drift. As gene flow is the strongest factor in restoring genetic variation of natural populations, genetic diversity in isolated laboratory populations will decrease continuously resulting in the loss of alleles. The level of random genetic drift depends largely on the number of individuals involved in reproduction. Thus, in population size-limited cultures, such as laboratory stocks, random genetic effects may alter the genetic structure and diversity within only a few generations. The *C. riparius* population which was monitored for 23 generations (Fig. 5.2) was maintained with an estimated effective population size (N_e) of 122.4 +/- 78.2 SD. This estimation was based on the assumption that each egg mass was produced by one unique male and female. Within this time span and in spite of maintaining a relatively high N_e in most generations, the population lost 29.5% of its initial level of

heterozygosity at the end of the experiment. The low degree of allelic richness within the investigated laboratory strains indicates a high level of inbreeding. Inbreeding has been shown to effectively affect several components of individual fitness, such as growth rates, fertility or juvenile mortality (Frankham et al., 2002). In addition, recent studies show that reduced fitness due to inbreeding, namely inbreeding depression, can be more severe under stressful conditions. For example, it has been shown that inbred lineages of *Drosophila melanogaster* were more sensitive to acetone and drought stress (Dahlgaard & Hoffmann, 2000) as well as copper sulfate and methanol exposure than outbred strains. Armbruster & Reed (2005) showed in a metaanalysis of 34 studies, that there is a general tendency for an enhanced sensitivity of inbred populations to environmental stress. These findings have severe implications on ecotoxicological research, as different levels of inbreeding could lead to biased results in bioassays which investigate life history responses of model species in the laboratory. In addition, different levels of inbreeding and thus genetic variability could explain variation between bioassay results and thus be responsible for a lack of reproducibility in toxicity tests. Even under constant laboratory conditions and test organisms originating from the identical stock could result in different test outcomes due to the stochasticity of random genetic drift. Recent studies in our laboratory document a significant effect of the initial level of genetic variation both on the fitness and sensitivity to Cd stress of *C. riparius* laboratory strains (unpublished data, Carsten Nowak, Goethe-University, Frankfurt am Main, Germany).

In order to avoid any bias in bioassay results due to high degrees of genetic impoverishment and inbreeding depression, we recommend monitoring genetic diversity in laboratory stock populations. Although experimental conditions for ecotoxicological tests are widely standardized by international guidelines, no such rigorous recommendations are provided for test organisms. Recent advances in molecular genotyping techniques, such as DNA

barcoding, amplified fragment length polymorphism (AFLP) and microsatellites provide effective tools to measure patterns of genetic variation in populations used in exposure tests. Our results show that the refreshment of laboratory cultures with individuals obtained from other laboratories does not allow for maintaining sufficient levels of heterozygosity. Even the crossing of eleven strains from different laboratories did not lead to a genetic diversity level comparable to that observed in field samples. Therefore, only regular refreshments of laboratory stocks with samples obtained from the field will allow establishing variable populations in the laboratory. However, although stocks that were initially sampled in the field are significantly more diverse than those of laboratory origin, they were still far from the level measured in the 'direct' field samples. Unfortunately, regular sampling in the field is hampered due to problematic species identification in both *Chironomus* larvae and adults. However, molecular genetic determination methods that have been established for many taxonomically problematic invertebrate taxa like chironomids (Carew et al., 2003) can serve as simple and universally applied identification tools.

Our current research projects combine above mentioned genetic tools with toxicological approaches to assess the role of genetic variation for animals subjected to environmental stress (e.g. temperature and toxic substances). Molecular genetic tools for species identification within the genus *Chironomus* are presently developed and will be applied to natural and laboratory populations.

6 Variation in tolerance to cadmium exposure among genetically characterized laboratory strains of the midge *Chironomus riparius*

Abstract

Estimating of the extent of intraspecific variation in tolerance to contaminant exposure is important in order to explain variation in the outcome of toxicity tests and to predict the effects of chemical stress on natural populations of plants and animals. However, only few studies provide evident data concerning intraspecific variation in life-history traits caused by a differential response to chemical stress. In this study we compared the life-history response of six laboratory strains of the midge Chironomus riparius *to cadmium exposure in a full-life cycle assay. In addition, the level of genetic variation in all strains was measured at five variable microsatellite loci.*

Several significant differences in life-history traits among the strains were observed in the controls and in cadmium treatments. However, the extent of variation between strains was largest at moderate cadmium stress (0.5 mg Cd/kg dw). At increased Cd concentrations all strains showed similar levels of high mortality and reduced reproductive success.

All strains showed considerable levels of genetic impoverishment compared to field populations. Strains with low genetic variation showed reduced fitness in the controls and were more susceptible to Cd exposure. For instance, no reproductive success in the lowest Cd treatment was observed for the strain with the lowest level of genetic diversity (PTG). In contrast, this Cd concentration had no negative effects on life-history traits of more variable strains. We discuss these findings in regard to their consequences for life-cycle exposure tests with chironomids.

Introduction

Laboratory exposure assays are an important tool in order to investigate the impacts of toxicants on life-cycle traits of plants and animals. However, laboratory test cultures are completely isolated and remain at small population sizes over several generations (Nowak et al., 2007). As a consequence of isolation and inbreeding, caged laboratory cultures will decrease in their level of genetic variation (Woods et al., 1989; Nowak et al., 2007). In addition, artificial selection leading to adaptation to laboratory conditions may accelerate the process of genetic impoverishment in laboratory populations (Woodworth et al., 2002). The effects of laboratory rearing on the genetic structure of test strains have raised questions concerning the transferability of experimental findings in the laboratory to natural environmental conditions (Woods et al., 1989; Duan et al., 1997; Nowak et. al., 2007). Furthermore, genetic differentiation and impoverishment may lead to varying test outcomes that may hamper the comparability among the outcome of laboratory exposure tests (Baird et al., 1991; Duan et al., 1997). For instance, Baird et al. (1990) showed that there is considerable variation in sensitivity to cadmium stress in *Daphnia magna*. As this species reproduces mainly parthenogenetically, genetic differentiation and different levels of preadaptation to toxic stress have been discussed to explain these findings (Baird et al., 1991; Soares et al., 1992). For solely sexually reproducing species, however, comparative studies concerning intraspecific variation in stress tolerance are hardly found in the literature.

In this study, we investigated the extent of variation in life-history response towards cadmium exposure between six laboratory strains of the non-biting midge *Chironomus riparius* (Diptera: Chironomidae). *C. riparius* is frequently used in life-cycle assays for the assessment of water and sediment toxicity (Vogt et al., 2007a). Furthermore, high levels of genetic impoverishment and considerable genetic differentiation have been identified among laboratory stocks of this species (Woodworth et al., 2002; Nowak et al., 2007), thus

providing an appropriate model system for the assessment of variation in stress tolerance between laboratory populations. All strains were exposed to sediment-associated Cd in a life-cycle test. Besides the survey of developmental and reproductive endpoints, we analysed the levels of genetic variation among strains at five variable microsatellite loci. Specifically, we focused on the following questions: (i) Are laboratory strains of *C. riparius* variable in tolerance to cadmium exposure? (ii) Does the level of genetic variation within the populations explain observed variation in susceptibility to Cd? (iii) Are molecular analyses of genetic variation, like microsatellite analyses, useful for the identification of increased stress susceptibility of laboratory strains?

Materials and Methods

Origin of laboratory strains

Chironomus riparius stocks were obtained from six laboratories across Europe (Table 6.1). In order to allow for acclimatization to new laboratory conditions, strains were kept under constant conditions (20°C, day:night rhythm 16:8, humidity 70%) for at least two generations.

Table 6.1. Abbreviations and origins of the *Chironomus riparius* laboratory strains used for the experiments.

abbreviation	origin
AMS	University of Amsterdam, The Netherlands
FLH	ECT Ökotoxikologie GmbH, Flörsheim, Germany
FFM	J. W. Goethe-University, Frankfurt am Main, Germany
LYO	Cemagref, Lyon, France
PTG	University of Aveiro, Portugal
LEY	RIZA, Lelystad, The Netherlands

Genetic analyses

Levels of genetic variation in the laboratory strains were measured at five microsatellite loci as described in Nowak et al. (2006). Allelic variation was determined for 24

individuals per population. Briefly, DNA was extracted from L4 larvae using a standard CTAB method (Winnepenninckx et al., 1993). After PCR amplification, fragments were loaded on a CEQ2000 capillary sequencer. Fragment lengths were scored using the CEQ 8000 Genetic Analysis System (version 9) software (Beckman Coulter®). GenAlEx V6 software (Peakall & Smouse, 2006) was used to calculate population genetic parameters (observed and expected heterozygosity, number of alleles per locus).

Life-cycle experiments

Life-cycle experiments were performed with all strains following the OECD-Guideline 218 (OECD, 2004) with modifications described in Vogt et al. (2007a). Each strain was exposed to four nominal concentrations of sediment-associated cadmium (0.5, 1.0, 1.5 and 2.0 mg/kg dw). Four replicates were used for each treatment. Each replicate consisted of a 600 ml glass beakers (Simax, Czech Republic), containing 100 g spiked dry quartz sand as sediment (grain size 0.1 - 0.4 mm, Quick Mix Group, Osnabrück, Germany) and 400 ml reconstituted water (pH-value 7.9 - 8.4; conductivity 540 µS/cm). The sediment was spiked with cadmium sulphate hydrate ($3CdSO_4 * 8H_2O$, Merck, Germany) solved in 300 ml EtOH per kg sediment (Merck, Germany, > 99.5% purity). A solvent control (SC) was included in all six parallel experiments. In each test vessel 20 first instar larvae were inserted using a stereo microscope (Olympus SZ 40, Hamburg, Germany). Test vessels were gently aerated through Pasteur glass pipettes, which were attached to an air compressor. Larvae were fed daily with a TetraMin® suspension (0.5 mg per larva, Tetra Werke, Melle, Germany). Emerged midges of each Cd-treatment were collected with an exhauster and transferred into a breeding container (30 x 20 x 20 cm) covered with gaze. A plastic dish (11.5 x 11.5 x 5.5 cm) filled with 400 ml of reconstituted water was provided for oviposition in every container. Egg masses were removed and counted daily. Number of eggs per egg mass was estimated according to Vogt et al. (2007a).

Following endpoints were considered in the experiments: larval mortality, mean emergence time (EmT_{50}), number of fertile egg masses per female and total number of fertile eggs per inserted larva ("reproductive output").

Statistical analyses

All data were analysed statistically using the software package GraphPad Prism® for Windows XP (Version 4.03, San Diego, USA). Significant differences between calculated means were determined with one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison post hoc test. For the calculation of mean emergence time (EmT_{50}) a non-linear regression was applied (cumulative logistic curve, variable slope) as shown in Vogt et al. (2007a). Only beakers with mortalities below 80% were considered for the determination of EmT_{50}. Life-history traits of the strains were correlated with the respective level of expected heterozygosity using Pearson correlation. In order to determine the impact of genetic variation on life-history, strains were pooled into two clusters with different heterozygosity (H_E) levels: (1) strains with highest H_E (AMS, FFM and LEY; "*high level*"), and (2) strains with lowest H_E (FLH, PTG and LYO; "*low level*"). Two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test was performed to determine the influence of genetic variation and cadmium-stress on life-history performance and to check for potential interactions between both factors.

Residual analysis of sediment and water

After the life-cycle experiments two sediment samples of each treatment were analysed for their Cd content at the laboratory of the International Graduated School Zittau, Germany (IHI) according to DIN EN ISO 17294-2:2005-02 guideline. For the analysis, approximately 100 mg sediment samples were decomposed using a mixture of 440 µl HNO3 (65%, subboiled), 200 µl H_2O_2 (30%, suprapur) and 100 µl HF (40%) within a microwave procedure (DIN norm 13656, MLS 1200 microwave, Lab Systems, Leutkirch,

Germany). Extracts were analyzed for Cd content using an inductive couplet plasma mass spectroscopy (DIN 38406, Elan 5000, Perkin Elmer, Überlingen, Germany). As reference materials standard soil GBW 08303 (polluted farmland soil, Bureau of Meteorology, Beijing, China) was analysed to test for extraction efficiency, showing recoveries of 97 to 100%. Detection limits were 0.015 µg Cd/l for the water phase and 7.5 µg Cd/kg dw for the sediment. Furthermore, the time weighted average test concentration was determined according to the OECD guideline 211 (OECD, 1998).

Results

Genetic analyses

Microsatellite analyses were successfully performed for 22-24 individuals of each laboratory strain. Observed heterozygosity (H_O) ranged from 0.0 (LYO) to 36.7% (AMS; Table 6.2). Similar values were calculated for the expected heterozygosity (H_E). No strain showed variation at more than three loci (data not shown). Number of alleles per locus ranged from 1.0 (PTG) to 1.8 (AMS, FFM, LEY). No significant deviations from Hardy-Weinberg equilibrium were detected at any locus after Bonferroni correction.

Table 6.2. Genetic variation in six laboratory strains of *Chironomus riparius* at five microsatellite loci. Shown are expected and observed heterozygosity (H_E and H_O), mean number of alleles per locus (N_A) and the number of individuals used in the analyses (n).

strain	n	H_E	H_O	N_A
AMS	24	0.314	0.367	1.8
FLH	24	0.102	0.092	1.2
FFM	24	0.285	0.235	1.8
LYO	22	0.101	0.127	1.4
PTG	23	0.000	0.000	1.0
LEY	22	0.305	0.282	1.8

Residual analyses

Calculated time weighted mean Cd concentrations are shown in Table 6.3. Recovery rates ranged from 58% (1.15 mg/kg dw) to 84% (0.42 mg/kg dw). Thus, calculated time weighted mean concentrations nearly represent the chosen nominal concentrations.

Table 6.3. Time-weighted mean concentration for the TBT and the Cd experiment according to OECD Guideline 211 (1998). SC = solvent control

nominal concentration	time-weighted mean concentration (± SD; *n*)
SC	0
0.5	0.42 (0.06; *2*)
1.0	0.74 (0.04; *2*)
1.5	1.02 (0.10; *2*)
2.0	1.15 (0.08; *2*)

Life-cycle test

Considerable variation between the strains was observed for several life-cycle traits. Control mortalities ranged from 10.0 to 46.3% (Table 6.4).

Table 6.4. Life-history traits of six *C. riparius* strains under Cd exposure. Significant differences between treatments and controls are indicated with asterisks (* = p <0.05, ** = p <0.01; one-way ANOVA with Dunnett's Multiple Comparison Test; [a] = significance level not calculable due to low number of replicates).

	control	0.42 mg Cd/kg	0.74 mg Cd/kg	1.02 mg Cd/kg	1.15 mg Cd/kg
mortality (in %; mean ± SD, *n*)					
AMS	10.0 (9.13, *4*)	22.5 (50.0, *4*)	66.3 (8.54, *4*)**	70.0 (15.8, *4*)**	96.3 (4.79, *4*)**
FLH	38.8 (14.4, *4*)	46.3 (18.9, *4*)	72.5 (22.2, *4*)*	90.0 (10.8, *4*)**	98.8 (2.50, *4*)**
FFM	36.3 (2.50, *4*)	57.5 (20.2, *4*)	85 (4.08, *4*)**	80 (12.9, *4*)**	96.3 (4.79, *4*)**
LYO	43.8 (20.2, *4*)	67.5 (16.6, *4*)	88.8 (11.1, *4*)**	86.3 (12.5, *4*)**	98.8 (2.50, *4*)**
PTG	15.0 (12.9, *4*)	97.5 (2.89, *4*)**	100 (0.00, *4*)**	100 (0.00, *4*)**	100 (0.00, *4*)**
LEY	46.3 (13.9, *4*)	42.5 (11.9, *4*)	60.0 (25.5, *4*)	75.0 (17.8, *4*)	85.0 (9.13, *4*)
mean emergence time (in d; mean ± SD, *n*)					
AMS	17.7 (1.00, *4*)	26.1 (1.08, *4*)**	31.2 (1.74, *4*)**	37.4 (5.51, *3*)**	nc
FLH	16.6 (0.84, *4*)	20.7 (0.61, *4*)*	23.7 (2.70, *3*)**	27.7 (0.00, *1*)	nc
FFM	17.3 (0.35, *4*)	22.5 (1.49, *4*)**	25.2 (0.00, *1*)[a]	34.4 (0.27, *2*)[a]	nc
LYO	15.7 (0.73, *4*)	20.5 (1.56, *3*)**	24.7 (0.00, *1*)[a]	23.0 (0.00, *1*)[a]	nc
PTG	17.3 (0.76, *4*)	nc	nc	nc	nc
LEY	16.1 (0.53, *4*)	21.7 (2.36, *4*)**	26.4 (1.75, *3*)**	24.5 (1.11, *2*)[a]	35.4 (2.30, *2*)[a]

All strains showed increased mortalities in all Cd treatments. However, in the lowest Cd treatment (0.42 mg/kg dw) significantly increased mortalities were only found in the PTG strain. In this strain, all but two (of 80 initially inserted) individuals died prior to emergence. Significant increased mortalities were observed in all strains in the higher Cd treatments except for LEY, for which a significant Cd effect was revealed only in the highest treatment (1.15 mg/kg dw).

Similar to mortality, a clear dose-response relation was found for the mean emergence time (EmT_{50}, Table 6.4). While EmT_{50} values were similar under control conditions, there was considerable variation between strains under Cd exposure. Highest variation in emergence time was found in the 1.02 mg/kg Cd dw treatment (14.4 days between LYO and AMS). However, all strains showed a significantly increased EmT_{50} compared to the controls in all Cd treatments. Because of high mortalities, EmT_{50} values could only be calculated for LEY in the highest treatment (1.15 mg/kg dw).

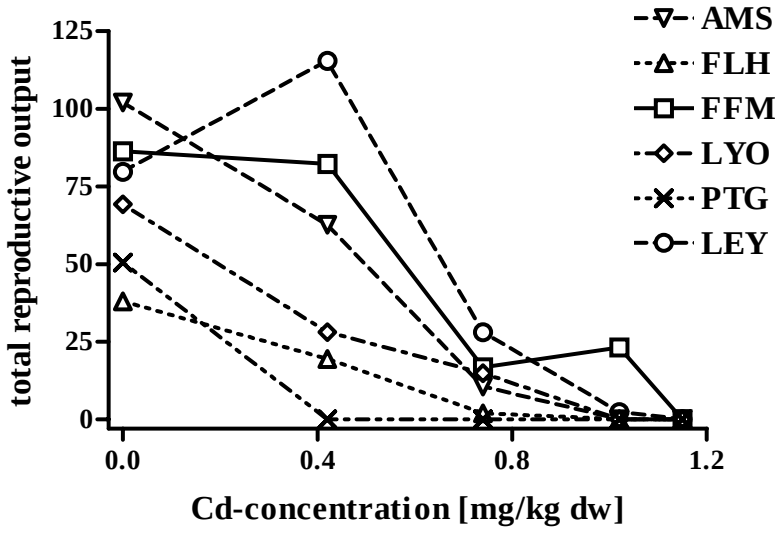

Figure 6.1. Total reproductive output (= number of fertile eggs per inserted individual) of six *Chironomus riparius* strains exposed to different Cd concentrations (for abbreviations see Table 1).

There was considerable variation in reproductive output between the strains both in the controls and under Cd exposure (Fig. 6.1). In the controls, total reproductive output ranged from 38 (FLH) to 102 (AMS) fertile eggs per introduced individual (Fig. 6.1). Reproductive output was significantly decreased at higher Cd concentrations (0.74 - 1.15 mg/kg dw; t-test, $p < 0.05$). The PTG strain produced no fertile egg mass in any Cd treatment. In contrast, all other strains successfully reproduced in the two lowest Cd treatments and reproduction was completely inhibited only in the highest Cd concentration (1.15 mg/kg dw).

Influence of genetic variation on life-cycle traits

No significant correlations were found between the level of genetic variation (H_E) and the mean emergence time or the number of fertile egg masses per female (Table 6.5).

Table 6.5. Pearson correlation between expected heterozygosity and mortality of six *Chironomus riparius* stocks at different cadmium concentrations (mg/kg dw); * = $p < 0.05$, ** = $p < 0.01$; SC = solvent control.

life-history trait	SC	0.42 mg Cd	0.74 mg Cd/	1.02 mg Cd	1.15 mg Cd
mortality	0.098	-0.799	-0.741	-0.961**	-0.668
EmT_{50}	0.253	0.695	0.847	nc	nc
fertile clutches/ female	0.781	0.647	0.461	-0.083	nc
reproductive output	0,8408*	0.9106**	0.7281	0.4208	nc

Mortality was not affected by the level of genetic variation in the controls. In contrast, strains with low diversity levels tended to show increased mortalities under Cd stress and a significant negative correlation between microsatellite variation and mortality was revealed for the 1.02 mg Cd/kg dw treatment ($p < 0.01$; Table 6.5). Significant correlations were

further revealed between H_E and total reproductive output under control conditions and in two Cd treatments (0.42 and 0.74 mg/kg dw).

Table 6.6. Two-way ANOVA results for four life-history traits of *Chironomus riparius*. Shown are degrees of freedom (df), F-values and significance levels (p). EmT_{50} = mean emergence time, fertility = mean number of fertile egg masses produced per female.

trait	effect	df	F	p
mortality	Cd x diversity	4	0.856	0.507
	diversity	1	7.566	0.012
	Cd	4	19.14	<0.001
EmT_{50}	Cd x diversity	2	0.837	0.459
	diversity	1	1.592	0.233
	Cd	2	22.51	<0.001
fertile clutches/ female	Cd x diversity	3	0.834	0.499
	diversity	1	5.079	0.042
	Cd	3	6.552	0.006
total reproductive output	Cd x diversity	4	7.688	<0.001
	diversity	1	31.18	<0.001
	Cd	4	37.34	<0.001

Two-way ANOVA revealed a significant influence of the level of genetic variation on both mortality and reproduction of the *C. riparius* strains (Table 6.6). Strains with low levels of heterozygosity ("*low level*"; PTG, LYO, FLH) showed slightly higher mortalities under Cd stress compared to the more diverse strains ("*high level*", AMS, FFM, LEY). However, No significant effects were detected in any treatment (two-way ANOVA with Bonferroni post hoc test; Fig. 6.2 A).

While fertility was clearly higher in the *high level* strains compared to the impoverished strains in the controls and the lowest Cd treatment, the influence of genetic variation on fertility disappeared under higher Cd stress (Fig. 6.2 C). The *low level* group had a nearly two-fold decreased total reproductive output compared to the more diverse strains in the controls ($p = <0.05$; Fig. 6.2 D). While this divergence between the *low level* and the *high*

level group increased under low Cd stress (0.42 mg/kg dw, $p < 0.001$), no significant differences between both groups were found at higher Cd concentrations.

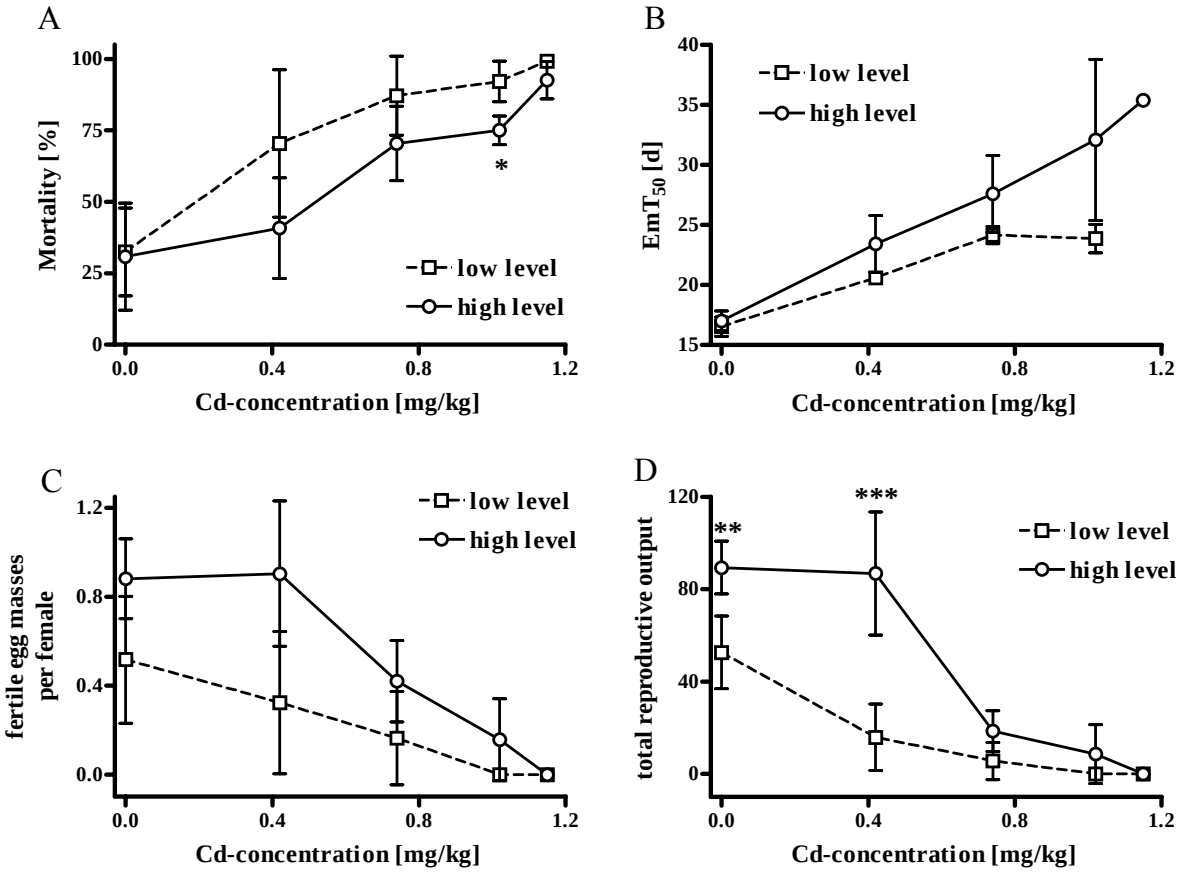

Figure 6.2. Life-history response to Cd exposure of *C. riparius* strains pooled into two groups with different levels of genetic variability. Shown are mortality (A), mean emergence time (EmT_{50}; B), number of fertile egg ropes per emerged female (C) and total reproductive output (D). "*Low level*" = strains with lowest levels of heterozygosity (n = 3, open square, closed line); "*high level*" = strains with highest heterozygosity values (n = 3, open circle, dotted line). Significant differences were calculated using two-way ANOVA with Bonferroni post hoc test (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Discussion

In the life-cycle tests cadmium had a negative effect on developmental and reproductive traits of all *Chironomus riparius* strains investigated. Considerable life-history variation was observed among strains under control and Cd-stressed conditions. Only two out of six

strains (PTG and AMS) showed control mortalities below 30%; thus only these tests can be regarded as valid following the OECD guideline 218 for *Chironomus* sediment toxicity tests (OECD, 2004). Even if we only consider the two valid tests, clear differences in tolerance towards Cd exposure become obvious. Mortality in the AMS strain was not significantly affected in the lowest treatment, while only 2.5% of all PTG larvae survived under this stress level. No larvae of the PTG strain survived in the higher Cd-stressed treatments. In contrast, emergence was still observed for AMS in the highest Cd concentration. High mortalities in the other four strains (FLH, FFM, LYO, and LEY) did not affect susceptibility towards the heavy metal. Although variation in mortality was observed between strains, none showed similar low tolerance as observed for PTG.

Reproduction was clearly inhibited by cadmium in all strains. However, there was high variation in reproductive output between the strains both in the controls (e.g., factor five between PTG and FFM) and under Cd stress. While PTG showed no successful reproduction even in the lowest treatment, FFM still produced fertile egg masses under high Cd exposure. Variation in reproduction is more difficult to interpret here, because no replicates were used for this parameter and thus no statistical evidences support observed variation in reproduction. In addition, mating may be less effective in small swarm sizes, which leads to lowered reproductive output (Armitage et al., 1995) per emerged female and thus bias the results observed in the high Cd treatments.

Unlike the mortality and reproduction parameters, mean emergence time (EmT_{50}) was very similar among strains in the controls, and clear variation in this trait among strains became obvious only in the presence of Cd. However, variation in all life-history traits among strains was highest under Cd exposure. Thus, observed variation is not only due to general variation in life-history, but also to variation in chemical tolerance. Variation in most traits tended to decrease at high Cd concentrations. At these high stress levels, all strains showed similar severe effects towards the chemical. The highest Cd-concentrations applied in this

study may have exceeded the general physiological potential of the species to cope with Cd stress. Therefore, genetic variation in stress response mechanisms (in the absence of long-term adaptation processes) will have strongest effects on life-history at intermediate stress levels (e.g., close to the LC_{50} for a certain species).

That considerable intraspecific variation in stress response towards chemical stress exists is well known for a range of animal and plant species. For instance, Baird et al. (1990, 1991) found that clonal lineages of the aquatic model species *Daphnia magna* showed variation in cadmium tolerance up to factor 100 between clones. Calculated LC_{50} values in this study varied between 0.22 and 1.40 µg/kg dw (based on nominal concentrations, data not shown), meaning a variation in stress tolerance of factor 6. These observations are in good agreement with other studies with concern to variation in chemical tolerance in molluscs (Forbes et al., 1995; Jacobsen and Forbes, 1997; Jensen and Forbes, 2001), collembolans (Crommentuijn et al., 1995), crustaceans (Naylor et. al., 1990) and amphibians (Bridges and Semlitsch, 2000). One explanation for this variation in stress response is genetic differentiation among source populations (Barata & Baird, 2002; Räsänen et al., 2003). Genetic variation is not evenly distributed in space, because migration barriers prevent unrestricted gene flow within the ranges of most species (Avise, 2004). Every isolated group of individuals has its independent evolutionary history and may evolve different levels stress tolerance due to random or selective processes (Barata & Baird, 2002; Rasänen et al., 2003). Accordingly, laboratory strains established from different geographical regions or ecologically divergent habitats may show different levels of stress tolerance which will lead to observed variation between tests.

An alternative explanation for this variation in life-history response under pollutant exposure is that different levels of genetic variation and inbreeding might affect fitness and stress tolerance of laboratory strains. It has been documented that *Chironomus* laboratory strains exhibit high degrees of genetic impoverishment (Woodworth et al., 2002; Nowak et

al., 2007). All six strains investigated here showed clearly reduced levels of genetic variation compared to field populations (mean H_E = 0.18 compared to 0.62 in two field populations, according to Nowak et al. [2007]). However, there was considerable variation in the degree of genetic impoverishment between the strains. The most Cd-susceptible strain, PTG, showed the lowest level of genetic variation. In this strain, no allelic polymorphism was detected at any locus. Significant correlations were found between the level of genetic variation and both mortality and total reproductive output of the strains under Cd-exposure. In addition, two-way ANOVA revealed that larval mortality and reproductive success in the life-cycle test were significantly affected by the level of genetic variation in the *C. riparius* strains. Thus, we found strong evidence that laboratory exposure assays with *C. riparius* are affected by the level of genetic variation within test strains. Over all, a clear trend was shown that strains with lower degrees of genetic variation display decreased fitness both under control and exposed conditions. The PTG example however indicates that lack of genetic variation may affect fitness of populations more severe in the presence of environmental stress. This observation is confirmed by a meta-analysis of 34 data sets on various animal and plant species (Armbruster and Reed, 2005). In this study it was shown that inbreeding depression is generally stronger in stressful environments. Inbreeding is unavoidable in small populations of caged organisms and the reduced level of genetic variation is most likely the consequence of the strong effects of inbreeding and genetic drift in small, caged laboratory strains without any gene flow from adjacent populations (Nowak et al., 2007). Inbreeding in *C. riparius* laboratory strains is thus most likely responsible for the negative correlations between heterozygosity and life-history reaction to Cd exposure observed in this study.

Although it has been expected that molecular techniques which measure genetic variation at neutral loci are not capable of indicating inbreeding effects in populations (Storfer, 1996), our results provide evidence for the usefulness of microsatellite analysis in genetic

investigations and inbreeding detection in laboratory populations. In order to prevent biased results due to strong inbreeding, microsatellite analysis could be used in order to monitor test strains of *C. riparius* regularly. Occasional refreshments from field populations, as suggested by Nowak et al. (2007), will help to maintain and effectively restore genetic variation in laboratory strains used for ecotoxicological tests.

7 Effects of environmental pollution on genetic diversity in natural populations of *Chironomus riparius* and *Chironomus piger*

Abstract

The idea that anthropogenic pollution results in reduced genetic variation in populations is often called the genetic erosion hypothesis. Although genetic erosion might be an important issue for the assessment of long-term effects of contaminants on populations, few studies have addressed this topic until present. In this study we investigated if environmental pollution affects genetic variation of natural populations of the sister species Chironomus riparius *and* C. piger *in the Rhein-Neckar region in Germany. We used DNA barcoding and cytotaxonomy for the identification of cryptic* Chironomus *larvae sampled at 39 sites in the study area. In addition five variable microsatellite markers were applied in order to discriminate between species and to assess patterns of genetic variation within and among populations.*

Results of DNA barcoding were consistent with the classically applied chromosomal analyses; thus our approach is useful for species identification of morphologically cryptic Chironomus *species. However, barcoding could not discriminate between* C. riparius *and* C. piger. *In contrast, Microsatellite analysis revealed clear differentiation among species and no evidence was found for interspecific hybridization. Genetic variation within* C. riparius *was correlated with various ecological characteristics recorded at the sampling sites. However, we found no clear evidence for the influence of any ecological parameters, including heavy metal contamination of sediments and life-history response of* C. riparius *to field sediment exposure, on genetic variation in populations. Furthermore, we found only weak differentiation between populations and accordingly high rates of gene flow among sites. Thus, any local effects of contamination on genetic variation of* Chironomus

populations will most likely be counterbalanced by high gene flow between populations in this area.

Introduction

Genetic diversity has been recognized to be a crucial factor for the long-term survival of natural populations (Brook et al., 2002; Frankham et al., 2002). Allelic variation provides the substrate for selection and thus enables populations to adapt to changing environmental conditions. Furthermore, loss of genetic variation leads to increased genome-wide homozygosity and populations with small effective population sizes are often subjected to reduced fitness due to inbreeding depression (Frankham et al., 2002). Although the role of inbreeding and reduced genetic variation is debated controversially in the literature (Amos and Balmford, 2001; Brook et al., 2002; Keller and Waller, 2002; Westemeier et al., 1998), loss of genetic diversity is widely considered to play an important role in extinction processes (Brook et al., 2002; Jamieson et al., 2006; Spielman et al., 2004). In addition, genetic variation has been shown to influence species composition of communities (Vellend, 2006). Regardless of this, intraspecific genetic diversity widely lacks consideration in many fields of biodiversity research. Ecotoxicologists, for instance, have just begun to investigate possible interactions between intraspecific variation and pollution stress (Belfiore and Anderson, 1998; Bickham et al., 2000). For instance, environmental contamination has frequently been argued to reduce genetic variability of populations in polluted environments (Staton et al., 2001; van Straalen and Timmermans, 2002; Whitehead et al., 2003). However, few studies were performed with the scope of testing for or against this 'genetic erosion hypothesis' in nature. Most field studies investigating patterns of genetic diversity in polluted areas lack a general investigation of background factors which affect genetic variation in natural environments (van Straalen and Timmermans, 2002). Thus, the question if environmental pollution leads to genetic erosion

of contaminant-exposed populations remains unanswered to date (van Straalen and Timmermans, 2002).

In this study we investigated if sediment pollution affects genetic variation in *Chironomus* populations. Chironomids are one of the most frequent and species rich taxa in aquatic habitats (Oliver, 1971). Chironomid larvae are of great importance for the function of aquatic ecosystems because of high decomposing rates in freshwater sediments, and they are a major food resource for numerous aquatic predators (Armitage et al., 1995; Michailova, 1989). Although chironomids play a key role in aquatic ecosystems and scientists have studied systematics and biology of this family in detail (Armitage et al., 1995), little is known concerning the genetic structure within and among chironomid populations and the factors which affect genetic variation in the field. One reason for the general lack of studies concerning ecology and population structure of chironomid species is the rareness of morphological traits which might be useful for species identification (Fittkau, 1966; Sharley et al., 2004). Larvae of the genus *Chironomus*, in particular, are morphologically cryptic (Pfenninger et al., 2007) and no taxonomic keys exist which allow for reliable species identification in this group. Solely microscopic analysis of the structure of salivary gland polytene chromosomes allows for relatively save species identification of larvae from this group (Keyl & Keyl, 1959; Michailova, 1989; Strenzke, 1959).

However, this technique requires expert knowledge and is too laborious for population studies which require species identification of hundreds of individuals (Pfenninger et al., 2007; Sharley et al., 2004).

Here, we chose a DNA barcoding approach (Hebert et al., 2002) for molecular discrimination of cryptic *Chironomus* species on a regional scale. We focused on a 40 x 60 km area of the upper Rhine valley plain (Rhein-Neckar-Region, Fig. 7.1). This region is an ancient cultural landscape, with intensive agri- and horticulture since Roman times. It is densely populated, including the major towns Mannheim, Ludwigshafen and Heidelberg,

all centres of various industries for centuries. Therefore, a substantial and variable anthropogenic impact on freshwater habitats in this area could be assumed *a priori*.

For our purposes, barcoding of *Chironomus* larvae served as a tool for the fast identification of midges belonging to *'Chironomus thummi'* Kieffer. Two subspecies have been described within this taxon, referred to as *C. th. thummi* and *C. th. piger*. However, nowadays both presumed subspecies are mostly regarded as separate species, *C. riparius* Meigen and *C. piger* Strenzke. We first applied microsatellite analysis and COI sequencing in order to investigate potential hybridization between the species. Furthermore, we recorded numerous environmental data, including the type of water body and sediment and water quality. To check for contaminant exposure, we applied two different approaches. As sediment dwellers, chironomid larvae are susceptible to sediment contamination (Sharley et al., 2004; Watts & Pascoe, 1996). Therefore, we analysed sediments from all localities for their heavy metal content. In addition, we conducted a life-cycle test with a laboratory strain of *C. riparius* in order to investigate the relative toxicity of the respective sediments on chironomids.

Ecological data were correlated with the respective levels of genetic variation of *C. riparius* and *C. piger* in order to reveal their potential effects on genetic diversity of *Chironomus* populations. Furthermore, we investigated patterns of genetic differentiation and gene flow among populations of the species.

Our results stress the need for accurate species determination and document that the assessment of population genetic patterns within and among populations, like genetic differentiation and gene flow, is crucial for accurate investigations of contaminant effects on genetic variation in natural populations.

Methods

Study area and sampling procedure

The sampling area lies in the middle of the upper Rhine valley in a rectangle of about 40 to 60 km between 49°09’ - 49°33’N and 8°10’ - 8°13’E. It comprises the Rhine valley plain, in the west limited by the mountains of the Pfälzer Wald and in the East by the rising hills of the Odenwald range. The area is hydrologically characterised by the presence of many drainage ditches, slowly flowing small streams, temporary puddles, the oxbows and the main stream of the river Rhine. The sampling took place from mid September to November 2004, thus sampling the over-wintering generation of *Chironomus* larvae (Armitage et al., 1995). Thirty-nine sampling sites were chosen opportunistically within the area, taking care to cover the area evenly (Fig. 7.1, Table 7.2).

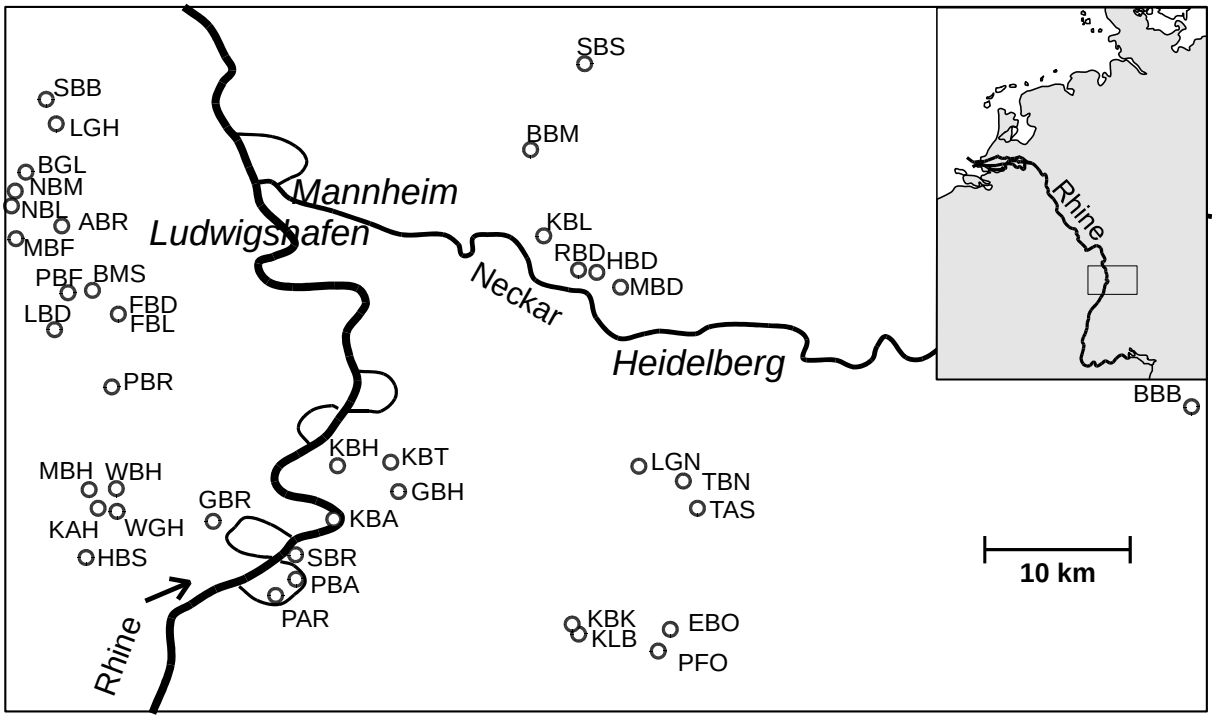

Figure 7.1. Distribution of sampling sites in the Rhein-Neckar region in Germany.

The different habitats present (puddles, ditches, streams and oxbows) were sampled approximately representative according to their abundance in the landscape. An area of 1x1 m was sampled with a 30 x 40 cm net of 0.5 mm mesh size. All instar stage 4 *Chironomus* larvae (L4) found, as identified by the presence of ventral tubules, were brought alive into the laboratory.

Species determination of field samples

In order to determine cryptic *Chironomus* species sampled in the study region, a DNA barcoding approach was chosen. The detailed procedure is found in Pfenninger et al. (2007). In brief, head and first body segments were removed from sampled larvae for polytene chromosome analysis. DNA from remaining caudal tissue was extracted using a standard CTAB protocol (see Nowak et al., 2006). Mitochondrial cytochrome oxidase I (COI) fragments were amplified on a Tetrad PTC-225 thermocycler performing initial five steps with 0.5 min 92° C, 0.5 min 48° C and 1 min 72° C followed by 35 steps with 1 min 92° C, 1 min 72° C and 1.5 min 72° C. Reaction mix contained 0.2 mM dNTPs, 3 mM $MgCl_2$, 1 x reaction buffer (20 mM Tris-HCl, 50 mM KCl; Invitrogen), 0.3 µM of each *Chironomus* specific primer (forward: 5´ TCG AGC AGA ATT AGG ACG ACC, reverse: 5´ AGG ATC ACC CCC ACC AGC AGG) and 1 U *Taq* DNA polymerase (Invitrogen) in a total volume of 10 µl. Approximately 40 ng of column purified (E.Z.N.A. Cycle-Pure Kit, Peqlab) DNA was used for cycle sequencing with the DTCS QuickStart sequencing kit (Beckman Coulter) following the instructions of the supplier and read automatically on a CEQ2000 capillary sequencer of the same manufacturer. Sequences were aligned using ClustalW (Thompson et al., 1994) followed by manual corrections. Sequences were collapsed to haplotypes and deposited in GenBank (accession numbers DQ910547 - DQ910729).

Obtained sequences were assigned to molecular operational taxonomic units (MOTU) as described in Pfenninger et al. (2007). To visualise the results, an unrooted neighbour-

joining (NJ) phenogram was constructed with PAUP 4.10b (Swofford, 1998). Support of nodes by the data was estimated using the bootstrap approach (Felsenstein, 1985). MOTU were defined as least inclusive terminal groups with at least 90% bootstrap support, using 1000 bootstrap replicates. The specific identity of a subsample of 100 individuals was determined using polytene chromosome analysis, following the protocol of Keyl & Keyl (1959). Expert cytotaxonomical species determination was kindly performed by Wolfgang Wülker, Freiburg im Breisgau. COI sequences of the sample specimen were assigned to sequences of cytotaxonomically identified specimen using the program TaxI (Steinke et al., 2005). This program calculates sequence divergences between a query sequence (taxon to be barcoded) and each of a set of reference sequences defined by the user. The respectively first individual of each nominal species as identified by polytene-chromosome analysis was arbitrarily taken as the reference sequence against which all other (query) sequences were tested.

Microsatellite analysis

Microsatellite analyses were performed for 253 individuals, which were previously identified as *'Chironomus thummi'* using DNA barcoding (Table 7.2). Microsatellite fragments were amplified using a T3 thermocycler (Biometra, Göttingen, Germany) as described in Nowak et al. (2006). Amplified DNA fragments were diluted 1:25 prior to fragment length analysis (ALF sequencer, Pharmacia Biotech, Uppsala, Sweden) and alleles were scored using the ALFWIN 1.0 software (Pharmacia Biotech, Uppsala, Sweden).

Assessment of genetic differentiation and hybridization between C. riparius *and* C. piger

We applied a Bayesian clustering approach to assign individuals to species on the basis of the microsatellite data. This approach, implemented using the program STRUCTURE (Pritchard et al., 2000), uses a model-based clustering method for inferring population structure from multilocus genotype data and to simultaneously assign individuals to clusters. It also allows for the identification of hybrid individuals and estimates the fraction

of alleles that are derived from each species. We ran five independent runs at population numbers (*K*) of *K* = 1 (no structure), *K* = 2 (two genetic clusters), ... *K* = *10* using a burn-in period of 50,000 iterations and collected data for 10^6 iterations. Runs were performed without using any information regarding species identification.

Identified genotype clusters were assigned to species (*C. riparius* or *C. piger*) via polytene chromosome analysis as described above, following the chromosome-based species identification scheme in Keyl (1957). Factorial correspondence analysis (FCA) on multilocus genotypes was used in order to graphically represent the distribution of genetic variation within and between *C. riparius* and *C. piger* (GENETIX 4.04 software, Belkhir et al., 1996-2002).

In order to check for patterns in the mitochondrial *'C. thummi'* data, a nested haplotype network based on statistical parsimony (SP) was build using the program TCS, version 1.06 (Clement et al., 2000). The SP network was based on 187 '*C. thummi*' COI-sequences. The nesting design was manually overlaid on the network following the rules given in Crandall (1996) and Templeton (1998).

Assessment of population genetic structure within C. riparius *and* C. piger

Expected heterozygosity (H_E), observed heterozygosity (H_O) and the mean number of alleles (NA) were calculated for *C. riparius* and *C. piger* based on the microsatellite data. Chi^2 test was used to test for deviations from Hardy-Weinberg equilibrium at all loci. The level of genetic differentiation among populations (here defined as individuals of a species occurring at one sampling site) was estimated using analysis of molecular variance (AMOVA). AMOVA estimates the ratio of variance among populations to total variance (Φ_{PT}), which is analogous to F_{ST} and can be used to estimate the level of population genetic differentiation. Additionally, the number of migrants (Nm) was estimated for all pairwise sampling site comparisons. All population genetic parameters were calculated using GenAlEx 6 (Peakall & Smouse, 2006).

Ecological habitat characterization

In total, 22 ecological parameters were recorded from all sampling sites (Table 7.1.). Categorical habitat structure characteristics were recorded taking into account the abundance of macrophytes and its breadth and depth. The following physico-chemical parameters were recorded: conductivity, pH, water temperature and O_2 saturation were measured with a WTW Multi 340i multimeter at each sampling point. Ammonium, nitrite and phosphate concentrations were calorimetrically determined using Aquamerk® quicktests. Chloride, CaCO3 and nitrate concentrations were measured with colour tests (Merkoquant®). Stream velocity was measured with an AMR ALMEMO® device. From 38 sampling sites, approximately 2 kg of sediment were taken to the laboratory and frozen at –20°C. Prior to analysis, they were thawed and homogenised. For the determination of the organic content, measured as loss on ignition, approximately 30 g were dried at 60°C for three days and weighted. The samples were then muffled at 550°C for 4h and the percentage of weight loss was determined.

In addition, sediment samples were analyzed for their content of seven heavy metals (Table 7.1). To this end, an aliquot of each sample (10 g) was freeze-dried. Following, 2 ml nitric acid (63%, Merck, suprapur) and 1 ml hydrogen peroxide (35%, Merck, suprapur) were added to the dried sediment in PTFE-vessels and decomposed (microwave assisted system, MLS 1200 mega). After cooling down to room temperature the solutions were filled up to 50 ml with water and analyzed for element content by inductively coupled plasma mass spectrometry (Perkin Elmer Sciex ELAN 6000). Certified reference materials (CRM; NIST SRM 8704) and blank values were analyzed at regular intervals (one CRM value and one blank value per four samples). The results of these analyses were within the certified range.

Table 7.1. Variable sets recorded, their abbreviations, units reported and describing statistics.

variable set	variable	unit	mean	SD	min	max
heavy metals (n = 7)	Cr content	µg/g	51.92	17.90	10.50	104.10
	Ni content	µg/g	22.81	8.51	7.30	48.60
	Cu content	µg/g	44.74	48.95	6.20	250.60
	Zn content	µg/g	274.99	209.39	33.60	884.00
	As content	µg/g	23.32	28.45	2.10	129.20
	Cd content	µg/g	0.71	0.79	0.07	2.95
	Pb content	µg/g	69.41	69.45	15.00	341.00
habitat structure (n = 3)	presence of macrophytes	0 = present, 1 = few, 2 = many	1.32	0.84	0.00	2.00
	breadth of water body	0 = < 50 cm, 1 = < 1 m, 2 = > 1 m	1.89	0.39	0.00	2.00
	depth of water body	0 = < 50 cm, 1 = > 50 cm	0.26	0.45	0.00	1.00
physico-chemical parameters (n = 12)	pH		7.70	0.39	6.87	8.70
	conductivity	µS/cm	845.76	455.45	118.00	2030.00
	stream velocity	m/s	0.06	0.10	0.00	0.41
	water temperature	°C	9.25	2.88	4.40	15.50
	O_2	mg/l	6.93	2.62	0.60	11.80
	nitrate	mg/l	21.97	81.55	0.00	500.00
	nitrite	mg/l	0.16	0.18	0.00	1.00
	phosphate	mg/l	0.79	0.72	0.00	3.00
	ammonium	mg/l	1.07	1.73	0.00	7.00
	$CaCO_3$	mg/l	269.81	134.39	53.40	462.80
	chloride	mg/l	16.03	12.91	3.06	85.80
	carbon content	% weight	16.03	12.91	3.06	85.80

Chironomus *life-cycle test for the estimation of sediment toxicity*

In order to evaluate the effective toxicity of the sediment samples on *Chironomus* larvae, we exposed a laboratory strain of *C. riparius* to all sediment samples in a life-cycle experiment according to OECD guideline 218 (OECD, 2004) using three replicates per sampling site and six controls. Larvae were kept at 20°C at 16/8h light/dark rhythm in 500 ml beakers with 80 g of native sediment. Details of the experimental procedure can be found in Oetken et al. (2005, 2007).

Table 7.2. List of sampling sites. Shown are sampling site abbreviations, their geographical position, the number of *Chironomus* L4 larvae used for genetic analyses, species richness for each sampling site and number of larvae assigned to *C. riparius* and *C. piger*.

sampling site	latitude	longitude	no. of identified *Chironomus* larvae	no. of species identified	no. of identified *C. riparius* larvae	no. of identified *C. piger* larvae
ABR	49.4777	8.3207	12	4	1	1
BBB	49.3677	9.0267	12	3	7	5
BBM	49.5268	8.6142	13	2	5	7
BGL	49.5121	8.2977	13	2	2	10
BMS	49.4380	8.3392	12	2		5
EBO	49.2263	8.7006	6	2		
FBD	49.4363	8.3249	10	2		1
FBL	49.4246	8.3557	8	1	8	
GBH	49.3144	8.5307	10	2	7	3
GBR	49.2932	8.4151	5	2	2	
HBD	49.4499	8.6542	8	7	6	1
HBS	49.2730	8.3339	8	1	7	
KBA	49.2950	8.4909	5	2		
KBH	49.3296	8.4913	26	3	24	
KBK	49.2283	8.6405	7	3		
KBL	49.4719	8.6223	9	2		
KBT	49.3313	8.5248	19	6	1	2
KLB	49.2264	8.6429	7	4		1
LBD	49.4145	8.3165	12	1	2	10
LGH	49.5431	8.3168	23	2	5	17
LGN	49.3291	8.6814	7	3		
MBD	49.4408	8.6674	11	1	8	1
MBF	49.4710	8.2921	13	2	2	10
MBH	49.3033	8.3440	9	5		
NBL	49.4996	8.2911	13	4		7
NBM	49.4919	8.2894	9	3	1	6
PAR	49.2456	8.4579	10	4		
PBA	49.2582	8.4700	5	1		
PBF	49.4363	8.3249	12	2		9
PBR	49.3789	8.3512	14	2		10
PFO	49.2137	8.6936	11	2	1	6
RBD	49.4519	8.6422	13	2	9	4
SBB	49.5579	8.3099	15	4	4	2
SBR	49.2749	8.4628	12	6		1
SBS	49.5813	8.6471	21	1	21	
TAS	49.3025	8.7166	6	2		
TBN	49.3207	8.7079	10	2		
WBH	49.3145	8.3361	6	4	2	1
WGH	49.3003	8.3542	11	2	8	
			Σ 433	ø 2.69	Σ 133	Σ 120

Correlation between genetic variation and ecological factors

Pearson correlation was used in order to investigate potential associations between the level of genetic variation (H_O) in populations of both species and the recorded ecological factors. All data with the exception of categorical variables and pH were either $\log_{10}$

(x + 1; continuous variables) or arcsin (percentages) transformed to conform to the underlying assumptions of normality and heteroscedasticity in subsequent analyses. In order to prevent bias in heterozygosity estimations due to low number of individuals, only sites with at least five individuals of the respective species where taken into account for the correlations.

Results

DNA barcoding and species identification

The number of *Chironomus* larvae found at 39 sampling sites ranged from 5 to 26 individuals with a mean of 11.1 +/- SD 4.75 (Table 7.2).

In total 432 *Chironomus* larvae were investigated with DNA-barcoding. 78 out of 100 chromosome preparations were of sufficient quality for species identification. Cytotaxonomical analysis revealed the presence of seven chromosomal species: *C. thummi*, *C. bernensis*, *C. luridus*, *C. plumosus*, *C. nuditarsis*, *C. commutatus* and *C. dorsalis*.

Forward COI sequencing resulted in readable sequences of at least 416 bp in lengths. All 432 COI sequences were collapsed into 190 different haplotypes, which were used for subsequent analyses. The neighbour-joining tree of the pairwise sequence divergences showed nine terminal clades with at least 90% bootstrap support (defined as MOTU1-9, Fig. 7.2). The mean uncorrected sequence divergence within MOTU ranged from 0.004 (MOTU8) to 0.081 (MOTU6). Average uncorrected mean distance among two MOTU was 0.156.

All inferred MOTU harboured only a single polytene-chromosome identified species. Chromosomal *C. thummi* individuals appeared only in MOTU1, *C. plumosus* in MOTU2, *C. nuditarsis* in MOTU3, *C. luridus* in MOTU4, *C. commutatus* in MOTU6 and *C. dorsalis* in MOTU9. One of these chromosomal species, however, occurred in two MOTU: chromosomal *C. bernensis* individuals belonged to the highly divergent MOTU 7 and 8 (Fig. 7.2). It was therefore possible to conclude from these inferred MOTU on the

chromosomal species, but not necessarily *vice versa*. We did not obtain chromosome preparations for MOTU5. Within *'C. thummi'* it was not possible to identify two groups, which could be assigned to *C. riparius* and *C. piger*.

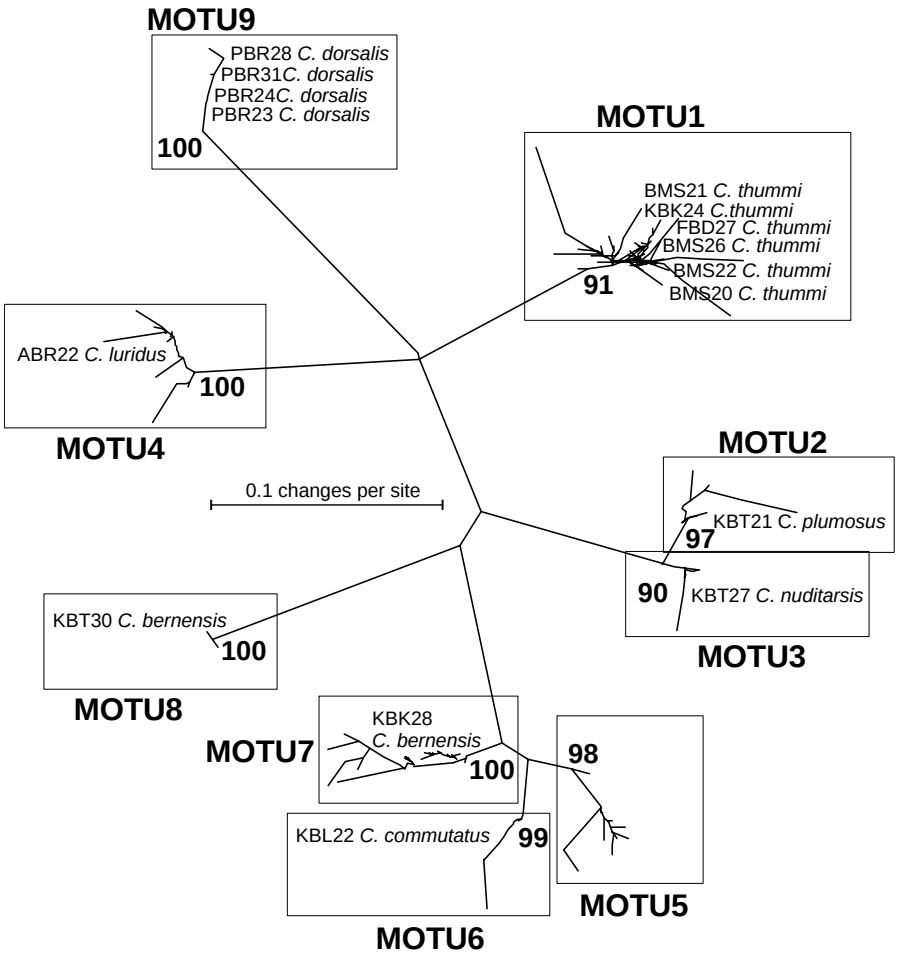

Figure 7.2. Neighbour-joining phenogram of ML-distances among *Chironomus* COI haplotypes. MOTU were inferred as least inclusive terminal groups with bootstrap values of 90% or more. Species names of cytotaxonomically determined individuals are provided.

Molecular identification of C. riparius *and* C. piger

Factorial correspondence analysis (FCA) based on microsatellite data from all individuals belonging to MOTU1 (*'C. thummi'*) is shown in Fig. 7.3. Two clearly separated clusters were identified. Comparison with cytotaxonomical analysis revealed that one cluster contained solely *C. riparius*, while individuals from the right cluster were all assigned to

C. piger. There was no evidence for intermediate multilocus genotypes indicating hybridization.

STRUCTURE outcomes were very similar to those obtained with FCA. Highest probabilities were obtained for K = 2, indicating the presence of two reproductively separated groups (data not shown). Furthermore, all but four individuals could be assigned two one of the groups with a probability of > 95%. The remaining four individuals could only be assigned to one group at a significance level of p <0.1. Comparison between FCA and STRUCTURE results showed a complete congruence in assignment of individuals to either *C. riparius* or *C. piger*.

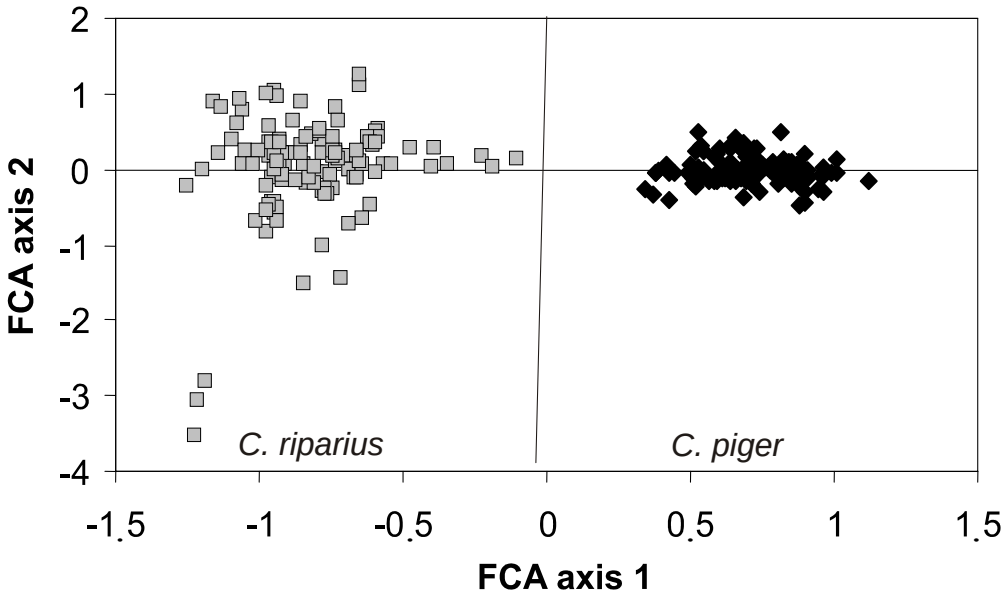

Figure 7.3. Factorial Component Analysis (FCA) of *'C. thummi'* based on microsatellite data.

The SP haplotype network based on 187 *'C. thummi'* sequences is shown in Fig. 7.4. In total, 68 haplotypes were identified which were nested in seven four-step haplotype clades. In order to test for congruence of nuclear and mitochondrial data, haplotypes were assigned to either *C. riparius* or *C. piger* based on microsatellite and chromosomal species identification. As shown in the figure, three haplotype clades contained only *C. riparius*

individuals, while four clades were assigned solely to *C. piger*. Thus, no discrepancies were found between nuclear and mitochondrial data.

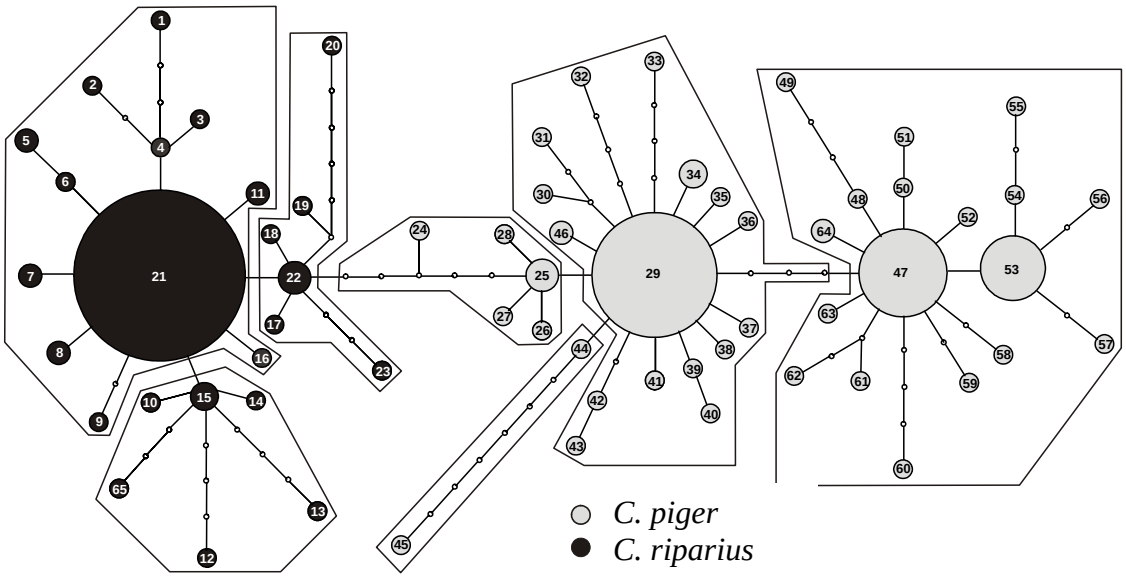

Figure 7.4. Nested clade diagram of *'C. thummi'* individuals based on 187 COI sequences.

Population genetic structure within C. riparius *and* C. piger

At 11 sampling sites five or more *C. piger* individuals were identified (Table 7.3). The mean number of alleles (N_A) at these sites ranged from 3.4 to 5.0, with an average of 4.2 over all sites. Heterozygosity values (H_O) ranged from 0.333 and 0.540, with an average of 0.432 over all sites. Similar values were found for H_E. Significant deviations from Hardy-Weinberg equilibrium were observed for two loci (MSC2 and MSC4, see Nowak et al., 2006) at five locations.

Genetic variation was found to be higher in *C. riparius* than in *C. piger*. Mean N_A was 4.7 and average H_O was 0.594 over all 12 sites with at least five *C. riparius* larvae identified. Significant Hardy-Weinberg deviations were revealed at two sites. Similar to *C. piger*, deviations occurred only at MSC2 and MSC4 locus.

Table 7.3. Genetic diversity of *C. riparius* and *C. piger* populations at sampling sites with n ≥ 5. Shown are number of individuals, expected and observed heterozygosity (H_E and H_O) and mean number of alleles per locus (N_A). Asterisks indicate a significant deviation from Hardy-Weinberg equilibrium at the loci shown in parentheses (see Nowak et al., 2006 for a detailed description of respective microsatellite loci).

species	site	n	H_E	H_O	N_A
C. piger	BBB	5	0.452	0.520	3.6
	BBM	7	0.447	0.405	3.6
	BGL	10	0.450	0.355*(MSC2)	4.6
	BMS	5	0.520	0.540	3.4
	LBD	10	0.478	0.500	4.4
	LGH	17	0.495	0.471*(MSC4)	4.8
	MBF	9	0.449	0.353*(MSC2)	4.4
	NBL	7	0.539	0.429	5.0
	PBF	9	0.451	0.422*(MSC2)	4.4
	PBR	10	0.488	0.420*(MSC2)	4.6
	PFO	6	0.353	0.333	3.4
	all sites	11	0.466	0.432	4.2
C. riparius	BBB	7	0.653	0.543	4.4
	BBM	5	0.564	0.640	4.4
	FBL	8	0.638	0.650	4.6
	GBH	7	0.575	0.533	3.8
	HBD	6	0.616	0.527*(MSC2)	4.4
	HBS	7	0.565	0.457	4.4
	KBH	24	0.669	0.616	5.8
	LGH	5	0.619	0.630	4.0
	MBD	8	0.694	0.775	5.4
	RBD	9	0.681	0.733	5.4
	SBS	21	0.605	0.577*(MSC2,4)	5.0
	WGH	8	0.555	0.450	4.4
	all sites	12	0.619	0.594	4.7

Analysis of genetic variation within and between sampling sites (AMOVA) revealed significant differentiation among sites for both *C. piger* and *C. riparius* (Table 7.4). However, global Φ_{PT} values were rather low (0.039 for *C. piger* and 0.046 for *C. riparius*). High rates of gene flow were identified among all locations. Calculated number of migrants (Nm) was > 1 in all pairwise comparisons of sampling sites for both species (data not shown). Average migration rate among all locations was Nm = 30.44 ± SD 40.18 for *C. riparius* and Nm = 5.91 ± SD 2.39 for *C. piger*.

Table 7.4. AMOVA results for *C. riparius* and *C. piger*. Shown are degrees of freedom (df), sums of squares (SS), percentages of variation among and within populations (% of variation), Φ_{PT} values, and significance levels (p).

species	source	df	SS	% variation	Φ_{PT}	p
C. riparius	among pops	11	58.250	5%	0.046	0.001
	within Pops	103	378.18	95%		
	total	114	436.43			
C. piger	among Pops	10	38.856	4%	0.039	0.011
	within Pops	85	244.74	96%		
	total	95	283.59			

Chironomus *biotest with native sediments*

Overall, larval mortalities were low in most field sediments (Fig. 7.5 A). Significant reduced survival rates compared to the controls were only revealed for HBD and KAH sediments. No sediment had a significant influence on sex ratio (data not shown). Mean emergence time (EmT_{50}) in the controls was 20 days for males and 22.5 days for females. No significantly delayed emergence was observed in any field sediment (data not shown). The average number of clutches produced per female was 0.9 in the controls and ranged from 0.4 to 1.2 in the treatments. Only in the SBB sediment, a significantly reduced egg production per female was observed (data not shown). A significantly reduced proportion of infertile egg masses were revealed for two sites (SBB, EBO).

Mean population growth rates (PGR) were 1.24 d^{-1} in the controls and ranged from 1.25 to 1.35 d^{-1} in all except two field sediments. Only sediments from EBO (1.17 d^{-1}) and SBB (PGR not calculable due to missing reproductive output) showed reduced population growth compared to the controls (Fig. 7.5 B).

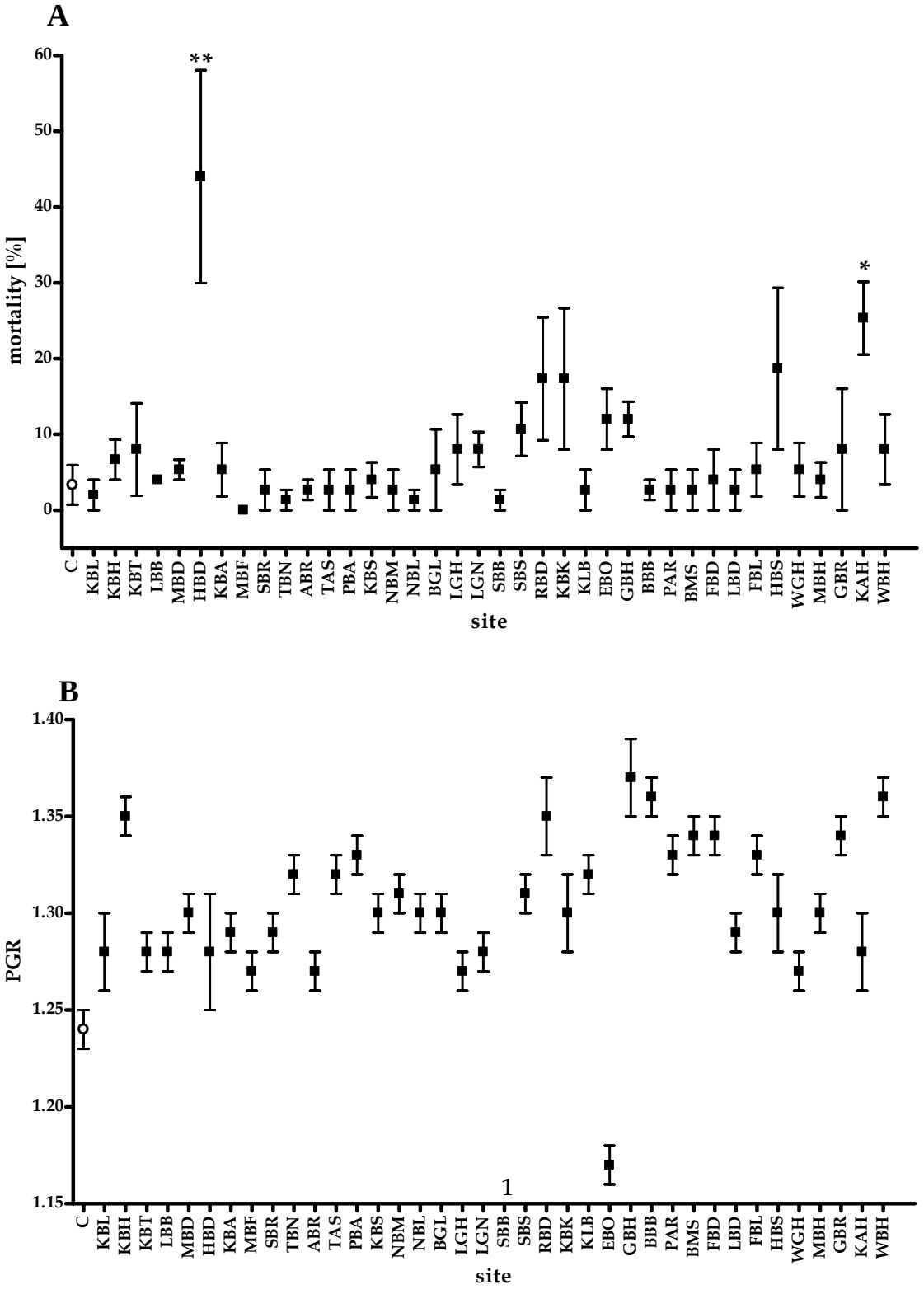

Figure 7.5. Mortality (± SEM; A) and population growth rate (PGR, in d^{-1}, ± SD; B) of a *C. riparius* laboratory strain exposed to 38 sediments taken from the sampling sites. C = control treatment with quartz sand. Significant differences between treatments and control were calculated using one-way ANOVA with Dunnett's post test. '1' indicates that no PGR was calculable, because no fertile clutches were produced in this treatment.

Correlations between genetic diversity and ecological parameters

Overall, correlations between ecological factors and heterozygosity levels were rather weak in both species. For each species, only one significant association was revealed. H_O in *C. riparius* was negatively correlated with nitrate concentration in the water (r = - 0.045, p = 0.045; Table 7.5). For *C. piger* a significantly negative correlation was found between heterozygosity and EmT_{50} values of the biotest (r = -0.802, p = 0.030). However, significances disappeared after Bonferroni correction for multiple comparisons.

Table 7.5. Correlation between heterozygosity (H_O) and habitat parameters. Shown are correlation coefficients (r) and significance levels (p).

	C. riparius		*C. piger*	
parameter	r	p	r	p
water breadth	-0.032	0.925	-0.115	0.806
water depth	-0.051	0.881	-0.371	0.413
macrophyte abundance	0.034	0.921	-0.110	0.814
pH	-0.207	0.542	-0.203	0.663
conductivity	-0.420	0.199	0.235	0.612
stream velocity	-0.075	0.827	0.176	0.706
water temperature	0.595	0.054	-0.693	0.084
O_2 content	0.049	0.886	0.454	0.306
nitrate	-0.613	0.045	0.164	0.725
nitrite	-0.286	0.393	0.473	0.284
phosphate	-0.462	0.153	-0.553	0.198
ammonium	-0.442	0.174	0.310	0.499
$CaCO_3$	-0.285	0.395	-0.422	0.346
chloride	0.100	0.798	0.119	0.799
carbon content	0.225	0.505	-0.267	0.563
Cr content	-0.123	0.720	0.029	0.951
Fe content	-0.433	0.183	-0.122	0.794
Cu content	0.085	0.804	-0.479	0.276
Zn content	0.487	0.129	-0.512	0.240
As content	0.583	0.060	-0.705	0.077
Cd content	0.356	0.282	-0.462	0.297
Pb content	0.257	0.445	-0.242	0.601
mortality	-0.221	0.514	0.120	0.797
EmT_{50}	-0.110	0.747	-0.802	0.030
sex ratio	0.115	0.737	0.564	0.187
clutch size	-0.311	0.353	0.439	0.324
fertile eggs/ female	0.166	0.625	0.547	0.204
PGR	0.183	0.590	0.654	0.111

Discussion

With the DNA-barcoding approach we could successfully cluster the identified haplotypes into well differentiated MOTU. Using the classically applied method for *Chironomus* species identification, the analysis of polytene chromosomal patterns, all but one MOTU (MOTU 5) could be assigned to known species. However, MOTU 8 and 9 were both identified as *C. bernensis*. We can not exclude the possibility of wrong species identification here, for some polytene chromosome preparations were of insufficient quality for safe species determination.

Overall we could show that DNA barcoding is a reliable method for the fast and safe identification of cryptic *Chironomus* larvae. The difficulty of species identification and the resulting low taxonomic resolution has been documented to hamper field studies on the ecology of chironomids (Butler et al., 2000; Wymer & Cook, 2003). DNA barcoding may provide a solution of this problem, allowing for field investigations with a high taxonomic resolution and without any taxonomical expertise needed. A more detailed discussion concerning the use of DNA barcoding of *Chironomus* species for the assessment of larval community structures of chironomids is found in Pfenninger at al. (2007).

The scope of this contribution, however, was to investigate population genetic patterns in the closely related sister species *C. riparius* and *C. piger*. Using COI barcoding we could successfully identify *'C. thummi'*, but we could not detect two genetically distinct groups that would allow for discrimination of the two presumed species. In contrast, the FCA plot based on microsatellite data of all pre-identified *'C. thummi'* individuals revealed the existence of two clearly separated groups within the taxon, which could be assigned to *C. riparius* and *C. piger* using polytene chromosomal analysis of a subsample of genotyped individuals. Both FCA and STRUCTURE analyses brought up no evidence for hybridization between the *C. riparius* and *C. piger*. Thus, at least within the investigated area, *C. riparius* and *C. piger* behave as 'good species'. The fact that both species do not

hybridize although they frequently occur sympatrically leads to the conclusion that effective isolation mechanisms must exist between the species. Although it has been documented, that species hybridize and produce viable hybrid offspring in the laboratory (Hägele, 1984), Hägele (1984, 1999) showed that pre- and postzygotic barriers exist between *C. riparius* and *C. piger* that may prevent hybridization in the field effectively.

In order to test if the two species are well differentiated in the mitochondrial genome, nuclear identified genotypes were plotted on a haplotype network based on mitochondrial COI sequences. As we showed, there was a clear differentiation between both species. However, genetic distances between both species were too low in order to distinguish between the species without any *a priori* information.

The level of genetic variation was high compared to that of all laboratory strains investigated in the previous chapters. Furthermore, very low rates of genetic differentiation and high gene flow among sites were revealed for both species. Chironomids have a rather limited active migration capacity (compared to other winged insects) of a few hundred meters during a life span (Armitage et al., 1995). However, passive transport has been frequently reported to be very effective in long distance migration, e.g., through wind dispersal (Armitage et al., 1995). Furthermore, *C. riparius* and *C. piger* occur very frequently within the investigated area and no obvious migration barriers which might prevent migration between populations exist within the study region. Low substructuring of genetic variation found in this study is thus in good agreement with *a priori* expectations of high gene flow between conspecific chironomid populations.

High gene flow rates are most likely contributing to the lack of ecological factors that were found to affect genetic variation of both species. Gene flow is very effective in maintaining and restoring genetic variation. Thus, reduced genetic variation in exposed populations will be effectively restored from migrants.

However, for *C. riparius* a significant negative correlation was found between the level of genetic variation and nitrate concentrations in the water, before Bonferroni correction was applied. High nitrate contents indicate strong eutrophication, which has frequently been shown to negatively affect species diversity in freshwater ecosystems (Armitage et al., 1995). Although nitrate itself is not toxic for freshwater organisms (Wetzel, 2001), eutrophic waters often show high levels of nitrite, which is an effective toxicant for many species (Wetzel, 2001). Furthermore, strong eutrophication leads to reduced levels of oxygen, which might most severely affect benthic organisms, like chironomid larvae (Wetzel, 2001). In our case, however, no significant effects of nitrite and oxygen content were found to influence genetic variation in *C. riparius* and thus the association found between nitrate concentration and reduced genetic variation remains unclear, particularly in regard to the fact that *C. riparius* is frequently found in highly organic polluted habitats (Rasmussen, 1984; Thienemann, 1954).

Nitrate content did not affect genetic variability in *C. piger*, but a significant association (before Bonferroni correction; see above) was revealed between heterozygosity in this species and mean emergence time in the life-cycle test. However, EmT_{50} was not significantly affected by any sediment in the test. Thus it remains unclear if this correlation indicates a functional link between genetic variation in *C. piger* populations and sediment toxicity or is rather due to Type I error, which may bias multiple correlations (Sokal & Rohlf, 1995). This hypothesis is confirmed by the application of Bonferroni correction, after which both significant results disappear. Overall, we thus found no hints for pollution induced genetic erosion in both species. The facts, that there is high gene flow between populations and that sediment toxicity was rather low at the sampling sites are most likely responsible for the observed lack of clearer effects of contaminant exposure on genetic variation in *Chironomus* populations.

Only few studies have actually provided strong evidence for genetic erosion in polluted environments (e.g., Kim et al., 2003; Kopp et al., 1992; Krane et al., 1999; Murdoch & Hebert, 1994; Street et al., 1998). Many others, however, found no correlation between genetic diversity in populations and the magnitude of exposure to xenobiotics (Baker et al., 2001; Brown et al., 2007; Chen et al., 2003; De Wolf et al., 2004; Larno et al., 2001; Muller et al., 2004; Nadig et al., 1998; Prus-Glowacki et al., 1999). Woodward et al. (1996), for instance, could not link levels of allozyme variation in *Chironomus plumosus* populations to a gradient of mercury contamination. As the authors themselves indicate, observed heterozygote deficiency was more likely due to a patchy population structure than to habitat contamination. Furthermore, this pattern might also be explained by a lack of taxonomic resolution in this study. *C. plumosus* is difficult to determine morphologically and several closely related species exist within the *C. plumosus* species group (Guryev & Blinov, 2002; Shobanov, 2005). Hence, observed deviations from Hardy-Weinberg equilibrium may be due to accidental consideration of two or more species in this study. The study of Woodward et al. (1996) thus provides a good example of the main difficulties that arise in field studies on genetic erosion. First, one has to take into account the variety of historical and ecological factors that affect patterns of genetic variation in nature. Secondly, high taxonomic resolution to the species level is essential in field investigations of population genetic patterns. The barcoding approach, combined with the analysis of nuclear multilocus DNA markers used in our study is a promising alternative to difficult morphological species determination and provides new opportunities for unbiased fine-scale studies concerning the genetic structure of taxonomically difficult organisms, like chironomid larvae.

8 General Conclusions

Biodiversity has been defined as:

"The variability among living organisms from all sources, including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems"
(Convention on Biological Diversity in Rio de Janeiro, 1992).

While species diversity has frequently been stressed to be crucial for the stability and health of ecosystems (Cardinale et al., 2006; Petchey, 2000; but see Hooper & Vitusek 1997), the importance of genetic diversity, here referred to as "diversity within species", for community and species persistence is controversially discussed in the literature (Frankham, 2003). It is one of the major goals of species conservation programmes to maintain or restore genetic diversity of endangered species, and the deleterious effects of genome-wide homozygosity on the fitness of organisms are well documented (Armbruster et al., 2000; Charlesworth & Charlesworth, 1987). Furthermore, loss of genetic variation has frequently been thought of to reduce the potential of populations to adapt to changing environments (Frankham, 1999). However, the role of intraspecific variation for the long-term survival of species is widely unknown to date. The most impressing examples for the importance of genetic variation in the short term come from laboratory (Ball et al., 2000) and field (Ingvarsson, 2002; Westemeier et al., 1998) investigations of small and isolated populations that suffer from inbreeding depression. Vilà et al. (2002) for instance, showed that a single immigrant was sufficient to restore genetic variation and 'rescue' a small and isolated population of *Canis lupus* from the detrimental effects of inbreeding depression. Measuring the effects of genetic variation on the long-term survival of populations under changing environmental conditions is, however, more difficult and few evidence exist for

the beneficial impacts of intraspecific diversity on population survival in changing and stressful environments (Frankham et al., 1999).

This thesis provides new evidence for the importance of genetic variation for population persistence. In Chapter 4 it is shown that genetically impoverished and inbred *Chironomus* strains show not only reduced fitness, but also increased susceptibility to heavy metal stress. The results are further confirmed in the sixth chapter, in which the life-history of six laboratory strains of *Chironomus riparius* strains exposed to cadmium was investigated. That inbreeding depression can be more severe under stressful conditions has been revealed for a range of animal and plant taxa (Armbruster & Reed, 2005). The findings in this thesis and in other studies have important implications for conservation of endangered species in changing environments. At present and during the past centuries, anthropogenic changes have led to dramatic environmental alterations within short time spans. Habitat loss and fragmentation leads to decreasing population sizes in many endangered animal and plant species which have to cope with various man-induced environmental stress factors, like climate change and pollution stress (Frankham, 2002). Thus, the fact that population decline is linked to the loss of genetic variation (Frankham, 1996), and that impoverished populations are more sensitive to anthropogenic stress, as shown in this thesis, lead to the conclusion that rare and endangered species will suffer more severely from presently occurring fast environmental changes than previously expected.

The other main finding in this thesis is described in Chapter 3. Within only a few generations, chemical exposure led to a significant decrease of genetic variability in *C. riparius* strains in the laboratory. This fast genetic impoverishment is explained by increased larval mortality and a reduced reproductive output, leading to lowered effective population size in stressed populations. As indicated in Chapter 7, however, overall high genetic variation was found in field populations of *C. riparius* and *C. piger* and genetic variation was not clearly affected by environmental contamination. *C. riparius* is a widely

distributed species occurring in large populations and high migration among *Chironomus* populations was observed in this study. This high gene flow preserves high levels of allelic variation among populations and prevents genetic erosion at contaminated sites.

The findings presented in this thesis show that genetic erosion due to environmental pollution is most relevant for small and isolated populations, but can be most likely neglected in widespread species with high dispersal capacities, like chironomids. Future studies seeking for genetic erosion in wild populations should therefore focus on species with patchy distribution patterns and limited migration potential.

Besides the investigation of genetic diversity in *Chironomus* populations in the field, intraspecific variation within and among laboratory strains of *C. riparius* was investigated (Chapter 5). High levels of genetic impoverishment compared to natural populations were found in all investigated laboratory strains. The previously in this chapter described finding, that reduced genetic variation and inbreeding affect tolerance to chemical exposure, are thus of general relevance for ecotoxicological risk assessment. In order to assess potential risks of xenobiotics for species and ecosystems, life-cycle exposure tests are of major importance in ecotoxicology. Although it is trivial to mention that laboratory experiments oversimplify the complex relations in natural ecosystems, the value of laboratory toxicity tests with highly impoverished strains is highly questionable, if laboratory organisms *per se* show a different response to chemical exposure than individuals of the same species in the wild. The findings in this thesis show, that laboratory exposure tests are indeed affected by the level of genetic variation in tests strains (Chapter 6), which has two major consequences for tests with this species and sexually reproducing organisms in general. First, the results of tests, which are performed with different strains, are not comparable among each other, because of different levels of genetic variation and inbreeding. Secondly, testing with laboratory strains will lead to continuous overestimations of chemical effects in natural populations. The microsatellite

markers, which are presented (Chapter 2) and applied in this thesis, are shown here to be useful for the identification of genetically impoverished *C. riparius* strains. Thus, regular monitoring of test strains with genetic markers will help to increase both comparability among tests and transferability of laboratory test results to natural conditions.

Outlook

The increasing use of molecular genetic techniques has provided new opportunities to investigate patterns of genetic variation within and among natural populations (Avise, 2004). Numerous studies document the effects of isolation, gene flow, drift and selection on genetic variation. The results presented in this thesis document the urgent need for investigating the consequences of anthropogenic environmental changes on genetic diversity of populations. The effects of environmental pollution on genetic variation and, *vice versa*, the effects of genetic variation of a population on its response towards contamination, could have unpredictable, reinforcing effects on population survival. Pollution stress lowers genetic variation, and lowered genetic variation leads to decreased susceptibility to chemical stress, which in turn may accelerate the rate of genetic erosion in pollution affected populations. Environmental pollution is bound to alter the adaptive landscape of species. Thus, ecotoxicology needs to consider both, short term effects of pollution, such as changes in life history traits of species, but also its long term effects, such as genetic erosion and the loss of evolutionary potential. Both effects are relevant to determine and to classify environmental threats.

The major threats to biodiversity, climate change, habitat destruction, biological invasions, overuse of genetic resources, and environmental pollution, do not appear solitary. Natural populations are faced to the combined effects of multiple anthropogenically induced environmental stressors. This thesis underlines the importance to investigate the effects of these rapidly occurring environmental changes on small and isolated populations, which are shown here to be more susceptible to environmental stress and genetic erosion.

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10 Zusammenfassung

Das Ausmaß an genetischer Vielfalt ist für Populationen in verschiedener Hinsicht von Bedeutung. Zum einen bildet genetische Diversität das evolutionäre Substrat für die Anpassung an sich ändernde Umweltbedingungen. Zum anderen geht eine Erniedrigung genetischer Diversität häufig mit Inzucht einher. Niedrige Heterozygotie und hohe Inzuchtraten wirken sich häufig negativ auf Überlebensraten und Reproduktion aus und erhöhen somit das Aussterberisiko von Populationen.

Die anthropogen verursachte Übernutzung von Flächen und die Fragmentierung ehemals zusammenhängender Areale, führen zur Bildung kleiner, voneinander isolierter Populationen. Da genetische Diversität positiv mit der Populationsgröße korreliert ist, sind solche kleinen 'Reliktpopulationen' häufig genetisch verarmt.

Neben Habitatzerstörung und -fragmentierung sind natürliche Populationen durch weitere anthropogene Einflüsse bedroht, wie etwa der Klimaerwärmung und der Verschmutzung von Luft, Böden und Gewässern. Es muß für den Biodiversitätsschutz folglich von großem Interesse sein, Interaktionen zwischen den genannten Stressoren und ihre Auswirkungen auf die Fitness von Populationen zu untersuchen.

Die vorliegende Arbeit beschäftigt sich mit den Konsequenzen reduzierter genetischer Diversität auf die Fitness von *Chironomus riparius*-Populationen unter Schadstoff-belastung. *C. riparius* ist ein Vertreter der weltweit verbreiteten und mit etwa 10.000 beschriebenen Spezies sehr artenreichen Familie der Chironomiden (Zuckmücken). Zuckmückenlarven dominieren im Benthos verschiedenster Gewässertypen hinsichtlich Artenvielfalt und Biomasse und stellen aufgrund ihrer Funktion als wichtige Stoffumsetzer und als Nahrungsquelle für viele Fische und Wasservögel eine Schlüsselgruppe in limnischen Ökosystemen dar. *C. riparius* wird des Weiteren in der Ökotoxikologie als Modellorganismus zur Bestimmung der Sedimenttoxizität nativer Sedimente, wie auch zur Risikoabschätzung von Chemikalien in standardisierten Biotestverfahren eingesetzt.

Um die Beziehungen zwischen Schadstoffbelastung und genetischer Variabilität in *Chironomus*-Populationen und zu untersuchen, wurden zunächst fünf variable Mikrosatellitenmarker für *C. riparius* entwickelt (Kapitel 2). Die Marker wurden auf den Polytänchromosomen der Speicheldrüsen von *C. riparius* lokalisiert, um eine mögliche physikalische Kopplung der Loci zu untersuchen.

In Kapitel 3 wird untersucht, ob Schadstoffexposition zu einer genetischen Verarmung von *C. riparius*-Populationen führen kann. Hierzu wurden zwei Laborpopulationen der Art über 12 Generationen dem hochwirksamen Pestizid Tributylzinn (TBT) in einer Konzentration von 160µg Sn/kg Sediment exponiert. Zwei weitere Zuchten wurden parallel auf unbelasteten Kontrollsedimenten gehältert. Die genetische Diversität wurde mittels Mikrosatellitenanalyse bestimmt; zusätzlich wurden in jeder Generation verschiedene Fitnessparameter (Larvalmortalität, mittlerer Schlupfzeitpunkt (EmT_{50}), Reproduktionserfolg) aufgenommen. Die genetische Variabilität nahm in beiden TBT-exponierten Populationen signifikant über die Zeit ab, während in den Kontrollansätzen kein derartiger Trend feststellbar war. TBT hatte weiterhin negative Effekte auf alle aufgenommenen Life-history-Parameter. Im Gegensatz zur genetischen Variabilität konnten jedoch keine zeitlichen Trends bei den Fitness-Merkmalen festgestellt werden. Es wurde jedoch eine große Variation in den Fitnessparametern sowohl zwischen den Populationen als auch zwischen verschiedenen Generationen beobachtet. Die Befunde zeigen, daß eine Belastung mit Umweltchemikalien die genetische Diversität von Populationen innerhalb weniger Generationen herabsetzen kann. Diese "genetische Erosion" wird bei seltenen und isolierten Populationen am stärksten sein und damit das Aussterberisiko von bedrohten Arten in belasteten Gebieten auf lange Sicht erhöhen.

Es ist in der Vergangenheit gezeigt worden, daß der Verlust genetischer Diversität die Fitness von Populationen erniedrigen kann. In Kapitel 4 wurde getestet, ob genetisch verarmte und ingezüchtete *C. riparius*-Populationen neben einer erniedrigten Fitness auch

eine erniedrigte Schadstofftoleranz zeigen. Hierzu wurden neun Laborzuchten der Art mit unterschiedlichen Diversitäts- und Inzuchtniveaus generiert und in einem Life-Cycle-Versuch an verschiedene Konzentrationen des Schwermetalls Cadmium exponiert. Die Ergebnisse zeigen, daß Inzucht und genetische Verarmung die Fitness von *C. riparius* sowohl unter Cadmiumbelastung als auch in den Kontrollen erniedrigt. Jedoch waren die Life-history-Unterschiede zwischen den Diversitätsniveaus unter Cadmiumbelastung insgesamt am größten. Zusätzlich konnte gezeigt werden, daß die Inzuchteffekte stark vom untersuchten Fitnessmerkmal abhängen. Während einige Parameter nur vom Inzuchtgrad abhingen, wurde für andere Fitnessmerkmale eine Interaktion zwischen dem Grad an Inzucht und der Stärke der Cadmiumbelastung festgestellt. So wiesen etwa alle neun Populationen unter Kontrollbedingungen eine ähnliche Larvalentwicklungsdauer auf, während hohe Cadmiumbelastungen den Emergenzzeitpunkt lediglich bei den stark ingezüchteten und genetisch verarmten Zuchten verzögerten. Diese Ergebnisse dokumentieren, daß die Faktoren Inzucht und genetische Verarmung Expositionsstudien mit Chironomiden stark beeinflussen. Da Inzucht und der Verlust genetischer Diversität in kleinen Populationen am stärksten sind, wird Schadstoffbelastung isolierte und lokal begrenzte Populationen stärker negativ beeinflussen als weitverbreitete und individuenreiche Populationen.

Um das Ausmaß an genetischer Verarmung in Laborzuchten von *C. riparius* zu dokumentieren, wurden 10 Zuchtstämme der Art aus verschiedenen Labors in Europa mittels Mikrosatellitenanalyse untersucht (Kapitel 5). Hierbei konnte gezeigt werden, daß alle Zuchtstämme gegenüber zwei Freilandpopulationen aus Südwestdeutschland hochgradig genetisch verarmt sind. Selbst durch die Kreuzung von 11 Zuchtstämmen aus Europa und den USA konnten kein dem Freiland ähnliches Diversitätsniveau erreicht werden. Des Weiteren wurde die genetische Variabilität einer Laborzucht über 23 Generationen im Labor untersucht. Dabei konnte eine signifikante Abnahme der

Heterozygotie schon nach wenigen Generationen festgestellt werden. Genetische Verarmung ist bei *C. riparius*-Zuchten im Labor durch die begrenzte Populationsgröße und eine völlige Isolation kaum zu vermeiden. Um eine Beeinflussung von Testergebnissen durch genetische Verarmung und Inzucht zu vermeiden, sollten Laborstämme von *C. riparius* daher regelmäßig mit Freilandindividuen aufgefrischt werden.

In den vorherigen beiden Kapiteln konnte gezeigt werden, daß genetische Verarmung die Reaktion von *C. riparius* gegenüber Schadstoffbelastung beeinflußt und daß Laborzuchten genetisch verarmt sind. In Kapitel sechs wurde daher getestet, inwieweit die Ergebnisse ökotoxikologischer Expositionsstudien bei Verwendung unterschiedlicher Testzuchten reproduzierbar sind. Hierzu wurden sechs verschiedene Laborstämme von *C. riparius* in einem Lebenszyklustest verschiedenen Cadmiumkonzentrationen ausgesetzt. Mittels Mikrosatellitenanalyse wurde das Ausmaß an genetischer Variabilität in den Zuchten untersucht. Signifikante Unterschiede in den aufgenommen Life-history-Parametern zwischen den Zuchten wurden sowohl in den Kontrollen als auch in den Belastungsgruppen gefunden. Die größte Variation zwischen den Zuchten trat jedoch unter moderatem Cadmiumstress auf. Bei hohen Belastungsgraden zeigten dagegen alle Zuchten stark erhöhte Mortalitäten und erniedrigte Reproduktionsraten. Die genetisch verarmtesten Zuchten wiesen im Gegensatz zu den diverseren Ansätzen schon unter Kontrollbedingungen eine erniedrigte Fitness auf und reagierten empfindlicher auf Cadmiumstress. So konnte etwa bei der genetisch verarmtesten Zucht (PTG) im Gegensatz zu allen anderen Ansätzen schon in der niedrigsten Belastungsstufe kein Reproduktionserfolg mehr beobachtet werden. Dieser Versuch zeigt, zusammen mit den Ergebnissen der beiden vorherigen Kapitel, daß Biotests zur Chemikalientestung mit Zuckmücken, wenn nicht gar mit allen sich sexuell reproduzierenden Organismen, durch Inzucht und genetische Verarmung in den Testpopulationen beeinflußt werden und ihre Aussagekraft für die Verhältnisse im Freiland in Frage gestellt werden kann.

Alle in den vorherigen Kapiteln dargestellten Ergebnisse wurden im Labor generiert. In Kapitel 7 wurde schließlich untersucht, ob die im Labor erhaltenen Ergebnisse auch auf die komplexe Situation im Freiland übertragbar sind. Hierfür wurden zunächst 432 in der stark anthropogen beeinflußten Rhein-Neckar-Region gesammelte *C. riparius* Individuen mittels DNA-Barcoding identifiziert und dann die genetische Diversität aller *Chironomus riparius* und *C. piger*-Individuen mittels Mikrosatellitenanalyse bestimmt. Es konnte hierbei keine Korrelation zwischen der genetischen Diversität in Populationen beider Arten und der Schadstoffbelastung an den jeweiligen Standorten festgestellt werden. Hierfür konnten zwei mögliche Ursachen identifiziert werden. Zum einen zeigte ein im Labor durchgeführter *Chironomus*-Biotest, daß Sedimentproben von den besammelten Standorten trotz recht hoher Schwermetallkonzentrationen kaum toxische Effekte auf *C. riparius* hatten. Des Weiteren wurde bei beiden Arten hoher Genfluß zwischen den Standorten festgestellt, welcher einer möglichen genetischen Verarmung an belasteten Standorten entgegenwirkt.

Wie diese Ergebnisse zeigen, ist die Untersuchung der Auswirkungen von Chemikalienstress auf die genetische Struktur von Populationen nur unter Berücksichtigung populationsdynamischer Prozesse sinnvoll. Das in dieser Arbeit im Labor beobachtete Phänomen der "genetischen Erosion" unter Schadstoffexposition sollte im Freiland insbesondere für kleine und isoliert vorkommende Populationen relevant sein. Isolierte Reliktpopulationen stellen jedoch die bevorzugten "Ziele" des Artenschutzes dar. Die beiden Haupterkenntnisse dieser Arbeit, daß Chemikalienbelastung sich negativ auf die Diversität kleiner, isolierter Populationen auswirkt und daß genetische Verarmung und Inzucht (die ja gerade in kleinen Populationen auftreten) die Sensitivität gegenüber Schadstoffexposition erhöht, haben für den Biodiversitätsschutz folglich eine große Bedeutung. Lebensräume von Tieren und Pflanzen werden in immer stärkeren Maße von anthropogen verursachter Landschaftszerstörung- und Fragmentierung isoliert, was zur Bildung zahlreicher kleiner und voneinander isolierter Populationen führt. Durch das Fehlen

von Genfluß zwischen diesen Populationen kann deren genetische Diversität durch anthropogenen Umweltstreß, wie Chemikalienbelastung oder der Veränderung des Klimas, herabgesetzt werden. Sollte der im Labor beobachtete Effekt, daß genetisch verarmte Populationen eine erniedrigte Toleranz gegenüber Umweltstress aufweisen, ein generelles Phänomen darstellen, so bedeutete dies eine noch stärkere Gefährdung der Biodiversität durch menschliche Einflüsse, als derzeit prognostiziert wird.

11 Curriculum and publications

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04/2003	Diploma degree in Biology at the J. W. Goethe-Universität Frankfurt am Main (1.0). Major subjects Ecology (1.0), Genetics (1.0), and Systematic Zoology (1.0)
06/2002 - 04/2003	Diploma thesis „Nukleäre und mitochondriale Phylogeographie des terrestrischen Gastropoden *Candidula gigaxii* Pfeiffer 1848 (1.1).
10/1997	Enrollment at the J.W.Goethe-Universität Frankfurt am Main. Field of study: Biology (Diploma).
11/1996 - 08/1997	Military service at the 2.Beob.Panz.Art.Lehrbat. 41 in Idar-Oberstein.
06/1996	"Abitur" at the Kaiserin-Friedrich-Gymnasium in Bad Homburg.

Publications

Articles

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* 1st prize for best oral presentation

** 1st prize for best poster presentation

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