Environmental change and its impact on hybridising Daphnia species complexes

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Environmental change and its impact on hybridising *Daphnia* species complexes

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Global change is mostly driven by the growing human population and constitutes a major challenge to the world’s biota. The general aim of this thesis project (Chapter 1) was to investigate the effects of human-made environmental change on the ecology, evolution and biogeography of hybridising Daphnia species complexes. The genus Daphnia comprises key species of aquatic food webs and constitutes a well-developed model in ecology, evolution and aquatic toxicology. The focus of the thesis was laid on three highly topical components of global change, i.e. eutrophication, micropollutants in aquatic systems and climate change. Together with my co-authors I report evidence for biological invasions, hybridisation and local species extinction associated with environmental changes in past decades and discuss potential threats due to on-going changes. This thesis also aims to contribute fundamental biogeographic data on past and present distributional patterns and describes the effects of environmental change in the context of ecological characteristics and distribution of species.

In Chapter 2 we reconstructed the invasion and establishment of European D. pulicaria in Lower Lake Constance from the resting egg bank in the lake sediments. We found evidence, that this invasion was facilitated by the eutrophication of Lower Lake Constance and were able to dissect population genetic processes of an invasion from the first colonisers to the establishment of the species in the lake. We propose a model of multiple introductions and subsequent monopolisation. This study represents an important contribution to the study of invasion genetics and highlights the significant influence of human-made ecological changes on the success of biological invasions.

Chapter 3 presents the results of an interdisciplinary approach of ecologists, environmental chemists and sedimentologists to study the impact of the continuing introduction of micropollutants into aquatic systems. In large scale ecotoxicological experiments, we studied the consequences of micropollutants detected in the sediments of Lake Greifensee on the ecologically and evolutionary important resting eggs of the D. longispina species complex from the same lake. We found significant effects on hatching success and hatching mortality and discuss mechanistic explanations as well as potential implications on the ecology and evolution of aquatic species that rely on a resting egg bank.

Knowledge of biogeography and ecological factors shaping the distribution of species are a prerequisite for the study of environmental change. In our phylogenetic analysis in Chapter 4 we showed that alpine lakes in the Pamir and Himalaya mountains host populations of D. longispina and D. dentifera. For D. dentifera in the Himalayan lakes we identified ecological factors explaining its
occurrence, showed that they can at least partly be related to avoidance of high UV conditions and identified a potential threat by the effects of rapid climate change on the hydrology of these lakes.

In Chapter 5 our paleogenetic study of two lakes south of the European Western Alps revealed biological invasions, taxonomic shifts and extensive hybridisation between members of the *D. longispina-galeata-cucullata* species complex associated with eutrophication and subsequent re-oligotrophication. We analysed the observed taxonomic succession in the context of known ecological characteristics of the species and trophic change and concluded that changes in food quantity and quality and size-selective fish predation pressure are the most plausible explanatory factors. We, furthermore, showed evidence that *D. longispina* also represented the native species in peri-alpine lakes south of the Alps. Finally, we propose the resting egg bank in Lake Varese as an outstanding model system to study the potential role of adaptive introgression in the evolution of the *D. longispina* species complex.

In conclusion, this thesis revealed strong impacts of past and on-going human-caused environmental change on the ecology, evolution and distribution of an aquatic key species. I summarise and discuss the implications of these findings in Chapter 6. Moreover, I highlight that the biological consequences of anthropogenic changes are not necessarily reversible by remediation measures and provide recommendations for future research.
Zusammenfassung


In Kapitel 2 rekonstruierten wir die Invasion und Etablierung der europäischen D. pulicaria von der Dauereierbank in den Seesedimenten im Bodensee-Untersee. Wir fanden Hinweise, dass die Invasion durch die Eutrophierung des Untersees begünstigt wurde und konnten die populationsgenetischen Prozesse im Zuge einer Invasion, von den ersten Kolonisten bis zur Etablierung der Art, im Detail studieren. Wir schlagen ein Model mit mehrfacher Einbringung von Kolonisten und nachfolgender Monopolisierung vor. Diese Arbeit ist ein wichtiger Beitrag zur Erforschung der genetischen Prozesse während einer Invasion und zeigt den Einfluss anthropogener Umweltveränderungen auf den Erfolg biologischer Invasionen.

Kenntnisse der Biogeographie und ökologischer Faktoren, welche die Artenverteilung beeinflussen, sind Voraussetzung für das Studium von Umweltveränderungen. In unseren phylogenetischen Analysen in Kapitel 4 konnten wir *D. longispina* und *D. dentifera* in alpinen Seen im Pamirgebirge bzw. im Himalaya nachweisen. Für *D. dentifera* konnten wir auch die ökologischen Faktoren, die das Vorkommen der Art beeinflussen, identifizieren und feststellen, dass diese Faktoren v.a. mit der Vermeidung hoher UV Strahlung in Zusammenhang stehen. Ausserdem fanden wir Hinweise für eine Gefährdung der Art in diesem Gebiet durch die Auswirkungen des Klimawandels auf die Hydrologie der dortigen Seen.


Chapter 1: Introduction and outline of the thesis

Anthropogenic global change, i.e. environmental changes caused by human activities that affect a substantial part of the globe, constitutes a major challenge for the world's biota. Pollution, eutrophication, climate warming and acidification as well as the introduction of species, fisheries, and hunting are well known examples for such changes caused by man. Global change has severe consequences for the evolution of species (Bradshaw & Holzapfel 2006; Hoffmann & Sgro 2011; Norberg et al. 2012) and their survival (Thomas et al. 2004). In line with that, it affects the occurrence and outcome of hybridisation and introgression between species (Rhymer & Simberloff 1996; Seehausen et al. 1997; Seehausen et al. 2008; Brede et al. 2009; Vonlanthen et al. 2012; Cahill et al. 2013). Therefore, humans are also considered as the world's greatest evolutionary force (Palumbi 2001).

In my thesis, I investigated the effects of human-caused environmental change on two hybridising Daphnia species complexes. Specifically, I focused on the impacts of eutrophication (Chapter 2, 4 and 5), micropollutants (Chapter 3) and climate change (Chapter 4) on the ecology, evolution and biogeography of the Daphnia longispina and Daphnia pulex species complex.

Selected examples of anthropogenic global change

Cultural eutrophication describes the enrichment of aquatic systems with nutrients as a consequence of human population growth, increasing industrialisation and agriculture. Eutrophication has become the primary problem for surface waters worldwide (e.g. Schindler 2006; Schindler 2012). The increased input of nutrients, in particular phosphorus and nitrogen, into lakes has severe effects on aquatic ecosystems. Among the most common outcomes of eutrophication are increased productivity, algal and cyanobacterial blooms, shifts in species composition and biodiversity, major food web disturbances (e.g. occasional fish kills) and water quality issues (see Correll 1998; Schindler 2006; Smith & Schindler 2009; Schindler 2012). Eutrophication can also facilitate the invasion of non-native species (Weider et al. 1997; Jankowski & Straile 2003; Chase & Knight 2006; Brede et al. 2009; Rellstab et al. 2011; Kokociński & Soininen 2012; Spaak et al. 2012) and affect the evolutionary trajectories and integrity of species, e.g. by promoting hybridisation and introgression (Seehausen et al. 1997; Seehausen et al. 2008; Brede et al. 2009; Vonlanthen et al. 2012). As a consequence, many European and several other developed countries, have taken measures to reduce the input of nutrients into aquatic systems. Governmental regulations, such as the ban of phosphorus in washing
agents, the installation of wastewater treatment plants and successful remediation measures have resulted in the restoration of the natural trophic state of several water bodies (e.g. Schindler 2012). Nevertheless, ecological and genetic legacies of these trophic changes are still detectable in the restored systems (e.g. Brede et al. 2009; Rellstab et al. 2011). In addition, for many aquatic systems, especially in developing countries, eutrophication constitutes an on-going issue awaiting realisable solutions (Nyenje et al. 2010; Vörösmarty et al. 2010; Pernet-Coudrier et al. 2012; Schindler 2012).

Although several developed countries have found appropriate solutions for the problem of eutrophication, a new threat to aquatic bodies has been emerging in the recent decades. Thousands of chemical compounds used in households, industry and agriculture are introduced into water bodies and represent a potential threat to aquatic organisms. These contaminants enter natural waters via different ways, e.g. waste water treatment plants effluents, urban and industrial sewage, surface runoff, spray drift and leaching from agricultural areas. Usually, these compounds, also referred to as micropollutants, are detected in low concentrations. Nevertheless, many of them are of toxicological concern and the variety of substances, their unknown behaviour in complex mixtures and the lack of knowledge about their fate in aquatic systems render them a potential and unpredictable risk to aquatic biota (Schwarzenbach et al. 2006; Sumpter 2009).

The probably best-studied example for global change is climate change (IPCC 2007). The rising temperatures world-wide and the implicated consequences for global weather systems affect, among others, the distribution and phenology of species as well as their survival and constitute an important evolutionary force (e.g. Parmesan & Yohe 2003; Thomas et al. 2004; Perry et al. 2005; Bradshaw & Holzapfel 2006; IPCC 2007; Hoffmann & Sgro 2011; Cahill et al. 2013). The effects of climate change extend to water bodies situated in the most pristine areas in the world, e.g. the pole caps and the Asian mountain ranges, and threaten the existence of organisms in these extreme habitats (Cruz et al. 2007; IPCC 2007).

**The model system *Daphnia***

Among the organisms that are affected by eutrophication, pollution and climate change are species critical for freshwater pelagic food webs that belong to the genus *Daphnia* (Crustacea: Anomopoda; water fleas). *Daphnia* species represent a major food source for fish and invertebrate predators, are important planktonic grazers, and therefore are attributed as keystone species in lentic ecosystems (Lampert 2011). *Daphnia* spp. have been established as important model organisms in ecology, evolution and aquatic toxicology (Ebert 2005; Altshuler et al. 2011; Colbourne et al. 2011; Lampert 2011; Orsini et al. 2013) and have recently been adopted as one of 13 model organisms for biomedical research by the National Institutes of Health (NIH 2013).
One of the reasons for the success of *Daphnia* as a model organism is its mode of reproduction. Most *Daphnia* species switch between clonal reproduction (parthenogenetic cycle) during favourable conditions and sexual reproduction (sexual cycle) when environmental conditions are not ideal. The induction of sexual reproduction is mediated by e.g. crowding, changes in food level and photoperiod and results in the production of sexual males and resting eggs (Figure 1). Such resting or dormant eggs are enclosed in a protective case called ephippium (see Zaffagnini 1987; Ebert 2005; Lampert 2011). A certain fraction of the produced ephippia floats on the lake surface and may be dispersed by e.g. waterfowl (Figuerola & Green 2002; Figuerola et al. 2003), insects (Van de Meutter et al. 2008), wind (Vanschoenwinkel et al. 2008) or human activities (Havel & Shurin 2004; Stasko et al. 2012) to other water bodies. A portion of the resting eggs sinks to the bottom of the lake where they contribute to the resting egg bank (e.g. Brendonck & De Meester 2003; Gyllstrom & Hansson 2004).

![Figure 1: Life cycle of a cyclic parthenogenetic *Daphnia*. Drawing by Dita B. Vizoso, Fribourg University (from Ebert 2005).](image)

The resting egg bank plays a crucial role for the ecology and evolution of *Daphnia* spp. and many other aquatic invertebrates (for comprehensive reviews on egg banks see Hairston & Kearns 2002; Brendonck & De Meester 2003; Gyllstrom & Hansson 2004). A variable fraction of the active pelagial
population is recruited via hatching from the resting egg bank. This interdependence, also known as benthic-pelagic coupling, has significant effects on population dynamics and the evolutionary potential of *Daphnia* (Cáceres & Hairston 1998; Gyllstrom & Hansson 2004). In fluctuating environments, the egg bank facilitates the maintenance of co-existence of species and genotypes via the storage effect (Chesson & Warner 1981; Chesson 1983; Cáceres 1997). In addition, the egg bank allows to escape from unfavourable biotic (e.g. Slusarczyk 1995) or abiotic conditions (e.g. Hairston & Olds 1987) and reduces the extinction risk of local populations. The egg bank increases the evolutionary potential of species by preserving and increasing genetic diversity (Ellner & Hairston 1994; Hedrick 1995). Moreover, a functional egg bank buffers local populations against the establishment of immigrants and reduces invasibility (De Meester et al. 2002).

Ephippia that sink down to deep parts of lakes do not receive the necessary hatching stimuli, e.g. increasing temperature and light (Vandekerkhove et al. 2005), and are covered with sediments over time. Therefore, they constitute an unbiased archive of evolutionary successful lineages of past populations. Such chronologically deposited resting stages can be retrieved by sediment coring. Ephippia can be extracted from the sediment cores and DNA for population genetics analyses can be isolated from eggs. In some cases, individuals still hatch from resting eggs several decades back in time and can be used for experiments. Moreover, the sediments record a whole set of ecological parameters that can be read out to reconstruct past conditions (e.g. Weider et al. 1997; Limburg & Weider 2002; Brendonck & De Meester 2003; Brede et al. 2009; Rellstab et al. 2011; Orsini et al. 2013). Using this approach, ecological and evolutionary changes in aquatic organisms in response to changing trophic conditions (Weider et al. 1997; Jankowski & Straile 2003; Brede et al. 2009; Rellstab et al. 2011), food quality (Hairston et al. 1999a; Hairston et al. 2001), predation pressure (Cousyn et al. 2001; Kerfoot & Weider 2004), parasites (Decaestecker et al. 2007; Pauwels et al. 2007; Pauwels et al. 2010), and heavy metals (Kerfoot et al. 1999) were reconstructed.

In summary, the key role of *Daphnia* spp. in aquatic food webs, their particular life history features and the availability of chronological biological archives, our comprehensive understanding of their distribution and biology and the wealth of toxicological information in combination with the now available genomic tools predestine *Daphnia* as a model system to study the effects of environmental change (Colbourne et al. 2011; Orsini et al. 2013).

**Hybridising *Daphnia* species complexes**

For a long time, hybridisation and introgression have been perceived as processes counteracting speciation and adaptive divergence, in particular in zoology (Mallet 2005, 2007; Abbott et al. 2013). In addition, hybridisation has often been assumed to be rare and of no evolutionary significance
(Mallet 2005). Today, however, a change of paradigm is observed among zoologists and evidence is accumulating that hybridisation is relatively frequent among closely related species and constitutes an important evolutionary force also in the animal kingdom (Abbott et al. 2013; Mallet 2005, 2007; Seehausen 2004). Hybridisation may impede or reverse speciation by increasing gene flow and recombination, or strengthen species boundaries by reinforcement. It may also accelerate speciation, either directly by polyploid or homoploid speciation or indirectly by introgression of loci that may promote adaptive divergence, i.e. via adaptive introgression (Abbott et al. 2013). In particular, the role of introgressive hybridisation in transferring adaptive alleles and its potential role in adaptive radiations has raised a lot of attention recently (Seehausen 2004; Pardo-Diaz et al. 2012; The Heliconius Genome Consortium 2012; Abbott et al. 2013).

The genus *Daphnia* comprises a radiation of ca. 100 species and recent discoveries of cryptic lineages suggest that considerably more species exist (Adamowicz et al. 2009; Petrusek et al. 2012). In my thesis, I focused on two major *Daphnia* species complexes: the *D. longispina* and the *D. pulex* species complex. Both complexes are widely distributed in ponds and lakes, their members show ecological differentiation and hybridisation and introgression between species within each complex are known (Colbourne et al. 1998; Flößner 2000; Schwenk et al. 2000; Petrusek et al. 2008; Vergilino et al. 2011; Marková et al. 2013).

The *D. longispina* species complex shows a mostly Holarctic distribution with a focus on the Palearctic. It comprises at least seven species and several cryptic lineages (Schwenk et al. 2000; Taylor et al. 2005; Ishida & Taylor 2007a,b; Petrusek et al. 2008; Adamowicz et al. 2009; Ishida et al. 2011; Petrusek et al. 2012) and hybridisation is frequently observed where species occur in syntopy (e.g. Schwenk & Spaak 1995; Taylor et al. 2005; Keller et al. 2008; Brede et al. 2009). Multiple evidence for hybridisation, introgression and reticulate evolution (e.g. Wolf & Mort 1986; Schwenk & Spaak 1995; Spaak 1996; Gießler 1997; Jankowski & Straile 2004; Taylor et al. 2005; Brede et al. 2009; Gießler & Englbrecht 2009; Ishida et al. 2011) has been found. Nevertheless, all taxa remain genetically distinct species with considerable levels of mitochondrial and nuclear differentiation (Schwenk et al. 2000; Petrusek et al. 2008; Adamowicz et al. 2009; Ishida et al. 2011). In line with this observation, incomplete reproductive isolation of species via impaired sexual fitness of hybrids and temporal separation of sexual reproduction has been demonstrated (Spaak 1995a; Schwenk et al. 2001; Jankowski & Straile 2004; Keller & Spaak 2004; Keller et al. 2007). Moreover, there is substantial ecological differentiation between taxa in this species complex with respect to food quantity (Gliwicz 1990; Gliwicz & Lampert 1990; Weider & Wolf 1991; Weider 1993; Boersma & Vijverberg 1994a,b; Spaak et al. 2012) and food quality requirements (Gliwicz & Lampert 1990; Seidendorf et al. 2007), vulnerability to predation (Spaak 1995b; Spaak & Hoekstra 1995, 1997; Spaak
et al. 2000; Spaak & Boersma 2001; Declerck & Meester 2003; Spaak & Boersma 2006; Wolinska et al. 2007), spatial distribution (Stich & Lampert 1981; Weider & Stich 1992; Flößner 2000; Seda et al. 2007) and susceptibility to parasites (Wolinska et al. 2006, 2007). Nevertheless, hybrids are frequently produced locally (Schwenk & Spaak 1995; Spaak 1997) and are often intermediate with respect to morphological and ecological characteristics (e.g. Weider & Wolf 1991; Weider 1993; Schwenk & Spaak 1995; Spaak & Hoekstra 1995). Furthermore, they combine traits of parental species that provide them with superior fitness under specific environmental conditions, resulting in hybrid dominance, an observation that led to the formulation of the “temporary hybrid superiority” hypothesis (Spaak & Hoekstra 1995, 1997).

The situation is similar for the D. pulex species complex. Its taxonomy is complicated by hybridisation, introgression and polyploidisation (Colbourne et al. 1998; Weider et al. 1999a; Vergilino et al. 2009; Vergilino et al. 2011; Marková et al. 2013) and the complex comprises approximately seven species and around ten distinct mitochondrial lineages, respectively. In addition, transitions to obligate asexual reproduction are known (Hebert 1981; Colbourne et al. 1998; Weider et al. 1999a,b; Marková et al. 2013). Similar to the D. longispina species complex, there is evidence for ecological differentiation between taxa (Hrbáček 1959, 1977; Flößner 2000; Cáceres & Tessier 2003; Marková et al. 2007; Dufresne et al. 2011; Pantel et al. 2011; Cristescu et al. 2012).

**Daphnia and its role as invasive species**

Biological invasions are also a topical issue and often by themselves considered as a component of global change (Simberloff et al. 2013). For instance, the invasion rate of freshwater cladocera has increased 50,000 fold over background levels before humans became an important vector for propagules (Hebert & Cristescu 2002; Roman & Darling 2007). In accordance with this general trend, also members of both Daphnia species complexes have recently undergone range expansions and became invasive. Various Daphnia invasions have been reported from around the world (Havel et al. 1995; Hairston et al. 1999b; Duffy et al. 2000; Mergeay et al. 2005, 2006; Brede et al. 2009; Duggan et al. 2012; Spaak et al. 2012; Frisch et al. 2013). One prominent example is the invasions of D. galeata in several peri-alpine lakes during peak eutrophication, which was followed by hybridisation with native species and pervasive genetic changes in these populations (Brede et al. 2009). In another case a single asexual North American D. pulex x pulecara clone spread throughout Africa and rapidly replaced native species (Mergeay et al. 2006). Interspecific hybridisation with consequences for the evolutionary trajectories of taxa and replacement of native taxa are a frequently observed consequence of Daphnia invasions (Weider et al. 1997; Mergeay et al. 2005, 2006; Brede et al. 2009; Dufresne et al. 2011; Reestab et al. 2011).
Thesis outline

In Chapter 2 I reconstructed the successful invasion of a member of the D. pulex species complex into Lower Lake Constance from its resting egg bank in the context of eutrophication. I aimed to unravel the identity and origin of the invading species. Furthermore, I used the resting egg bank to investigate the population genetics processes during the invasion.

In the subsequent chapters, I focused on the D. longispina species complex. Another aspect of anthropogenic environmental change that has only recently been recognised is the introduction of micropollutants into the aquatic environment. Chapter 3 addresses this issue and describes an experimental study that aimed to identify potential ecotoxicological effects of micropollutants on the fitness of the resting egg bank of the D. longispina species complex.

In Chapter 4, I studied members of this complex in one of the most pristine habitats on this planet, the Pamir and Himalayan mountains. In order to assess the potential impacts of environmental change, knowledge on the biogeography and ecological factors determining the occurrence of taxa is indispensable. Therefore, I attempted to clarify the uncertain taxonomic position of these species and to identify the abiotic and biotic factors explaining their distribution in this harsh environment. Moreover, I discussed the potential implications of the rapid climate change in this area on the extinction risk of these species.

In Chapter 5 I return to the topic of eutrophication and its consequences for Daphnia. Again, I made use of the resting egg bank and reconstructed the effects of trophic changes on the taxonomic composition and patterns of hybridisation for three species in the D. longispina species complex. This study was conducted on lakes situated south of the European Western Alps, an important part of the species' geographic range for which information on past taxonomic assemblages was lacking.

Finally, in Chapter 6 I attempted to summarise the results of the four studies and give recommendations for future research on the topic.

References


Introduction


Chapter 2: A human-facilitated invasion reconstructed from the sediment egg bank using genetic markers

In submission to Molecular Ecology
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Abstract

Biological invasions and human-made ecological changes are global issues with far-reaching consequences for single species, communities and whole ecosystems. However, the role of anthropogenic ecological changes in invasions has received surprisingly little attention yet. Furthermore, understanding modes and mechanisms of biological invasions requires knowledge of the genetic processes associated with successful invasions. In many instances, this is particularly difficult as the initial phases of the invasion process often pass unnoticed. In this study, we combine historic information with a paleogenetic approach to reconstruct the invasion of a *Daphnia* sp. into Lower Lake Constance, starting during peak eutrophication in the 1970s, from its resting egg bank using genetic markers. We identified the invader as European *D. pulex* originating from meso- and eutrophic lowland ponds in Central Europe. The founding population was characterised by an extremely low genetic diversity in the resting egg bank that increased over time until it plateaued. Furthermore, strong evidence for selfing and/or biparental inbreeding was found during the initial phase of the invasion. We conclude that Lower Lake Constance was invaded by European *D. pulex*, pre-adapted to high trophic conditions in their native habitat, and that this invasion was therefore facilitated by human-caused eutrophication. Our genetic data are in agreement with a scenario of multiple introductions and subsequent monopolisation. In this context, we discuss the specific life history characteristics of *Daphnia* spp. that allow them to overcome genetic pitfalls during an invasion. Our study dissects the genetic processes at work during an invasion and highlights the significant influence of human-made ecological changes on the success of biological invasions.

Keywords: *Daphnia*, egg bank, ancient DNA, eutrophication, asexual, selfing, ND5, Rab4, LDH-A
Introduction

Biological invasions are globally a topical issue as they can have strong impacts on native species, communities and ecosystems, constitute a major threat to biodiversity and can cause high economic costs (e.g. Sakai et al. 2001; Simberloff et al. 2013). During several decades of biological invasion research many factors facilitating successful invasions have been identified. However, scientific effort has predominantly focused on the identification of properties of invasive species that could explain successful invasion. Recently, Simberloff et al. (2013) called for more attention as to how anthropogenic changes of ecosystems may facilitate invasions (MacDougall & Turkington 2005; Gaertner et al. 2012; Spaak et al. 2012).

Cultural eutrophication of aquatic systems, caused by human population growth, industrialisation and the resulting increase in nutrient inputs, fundamentally affects ecosystem processes (Correll 1998; Schindler 2006; Smith & Schindler 2009; Schindler 2012). Eutrophication constitutes the primary problem for the majority of surface waters around the world as it leads to increased biomass production, fish kills, reduction in species diversity, changes in species composition (e.g. Correll 1998; Smith & Schindler 2009), affects the evolution of species (Seehausen et al. 1997; Seehausen et al. 2008; Brede et al. 2009; Vonlanthen et al. 2012) and may also facilitate biological invasions (Weider et al. 1997; Jankowski & Straile 2003; Chase & Knight 2006; Brede et al. 2009; Rellstab et al. 2011; Kokociński & Soininen 2012; Spaak et al. 2012). Despite the introduction of measures to reduce nutrient inputs and successful restoration of the trophic state of many lakes, some of them still harbour the legacy of past eutrophication events in form of invasive species (Brede et al. 2009; Rellstab et al. 2011; Spaak et al. 2012).

Well-studied in the context of biological invasion and eutrophication are species of the genus Daphnia, which are key organisms in many aquatic food webs that can exert strong effects on the ecosystem (Lampert 2011). Recently several cases of invasions of Daphnia spp. have been reported (Havel et al. 1995; Hairston et al. 1999a; Duffy et al. 2000; Mergeay et al. 2005, 2006; Brede et al. 2009; Spaak et al. 2012; Frisch et al. 2013). However two of the most striking examples probably are: invasions of D. galeata in several peri-alpine lakes during peak eutrophication, which was followed by hybridisation with native species and pervasive genetic changes in these populations (Brede et al. 2009), and the invasion of a single asexual D. pulex x plicaria clone that spread throughout Africa and rapidly replaced native species (Mergeay et al. 2006). High propagule pressure and genetic diversity are considered to contribute significantly to invasion success (Sakai et al. 2001; Kolbe et al. 2004; Frankham 2005a,b; Lockwood et al. 2005; Roman & Darling 2007). Daphnia spp. have three essential properties that enable them to exert high propagule pressure and ameliorate negative
impacts of diversity loss during the invasion process (Kolbe et al. 2004; Frankham 2005a,b; Roman & Darling 2007) and therefore predestine them as successful invaders. First, they are able to reproduce asexually, either by cyclic or obligate parthenogenesis, which provides them with certain demographic advantages, i.e. avoidance of the “twofold cost of sex” and Allee effects and the ability to found a population starting from a single individual (Sakai et al. 2001; Mergeay et al. 2005, 2006; Roman & Darling 2007). It further allows them to circumvent inbreeding depression and to preserve successful genotypes (Vrijenhoek 1998; Sakai et al. 2001; Roman & Darling 2007). Second, Daphnia spp. produce ephippia, protective chitinous envelopes that enclose up to two resting eggs (Zaffagnini 1987; Lampert 2011). Ephippia are very resistant to harsh environmental conditions and can easily be distributed over long distances, e.g. through migratory water fowl, wind or human-mediated dispersal, and allow for high propagule pressure (De Meester et al. 2002; Lockwood et al. 2005; Roman & Darling 2007). Furthermore, a proportion of ephippia sinks down to the lake bottom and builds up a so called resting egg bank that reduces the extinction risk in the newly colonised habitat, i.e. storage effect (Chesson 1983) and preserves and increases genetic diversity (Hedrick 1995; Brendonck & De Meester 2003). Third, Daphnia spp. can quickly respond to ecological change and have a high adaptive potential that allows for rapid local adaptation and monopolisation (Cousyn et al. 2001; De Meester et al. 2002; Van Doorslaer et al. 2010; Colbourne et al. 2011; De Meester et al. 2011; Latta et al. 2012; Orsini et al. 2013).

Here, we combined paleogenetic investigations of the sediment egg bank with evaluating historical information on zooplankton abundance (Einsle 1987) to reconstruct population genetic processes during the invasion and successful establishment of a non-native Daphnia species (belonging to the D. pulex species complex) in a large European lake starting from its first appearance during peak eutrophication 40 years ago up to recent years (Figure 1). Daphnia egg banks have been successfully used already to reconstruct evolutionary processes and invasions (e.g. Hairston et al. 1999a,b; Duffy et al. 2000; Cousyn et al. 2001; Mergeay et al. 2005, 2006; Decaestecker et al. 2007; Brede et al. 2009; Rellstab et al. 2011). Nevertheless, this is the first time that an invasion comprising more than a single genotype (Mergeay et al. 2006) and not being obscured by hybridisation events (Brede et al. 2009; Rellstab et al. 2011) was investigated using high resolution markers to identify the invader and to unravel the genetic processes at work.
**Figure 1:** Patterns of eutrophication and re-oligotrophication in Lower Lake Constance over the last four decades. Changes in total phosphorus concentrations, a surrogate for trophic state, are provided separately for the two basins Gnadensee and Zellersee (© BOWIS - Daten aus dem Bodensee-Wasserinformationssystem der Internationalen Gewässerschutzkommission für den Bodensee, IGKB). The arrow indicates the time of first appearance of *D. pulicaria* according to Einsle (1987).

**Material and Methods**

**Sampling site and sediment cores**

Lower Lake Constance (LLC) represents the smaller and shallower part of Lake Constance situated along the border of Switzerland and Germany. The Lower Lake comprises three main basins named Gnadensee (including Markelfinger Winkel), Zeller See and Rheinsee. Sediment cores with a diameter of 105 mm were collected with a gravity corer from two different locations, i.e. Markelfinger Winkel (N47.7265445°/E9.0156945°; 17 m water depth) and Gnadensee (N47.7071778°/E9.0665917°, 21 m water depth), and stored in the dark at 4 °C until further processing. Three cores from each site were used for this study, cut into halves and sliced into 0.5 cm slices. In order to avoid smear contamination 2-3 mm of the surface and the outer margins of each core half were removed. One half core from each site was dated using $^{137}$Cs and $^{210}$Pb (Appleby 2002). All cores were aligned using the reconstructed age models and conspicuous sedimentological characteristics.
**Ephippia preparation and DNA extraction**

Sediments were sieved through 224 µm and 250 µm mesh size sieves and *Daphnia pulex* ephippia were selected under the stereo microscope, counted and washed with autoclaved nanopure water. Each ephippium was placed in a single drop of autoclaved water and flipped open with sterilized dissection needles. Eggs were assigned a quality value ranging from 1 (good quality) to 4 (bad quality) based on their coloration and transferred into single 200 µL tubes containing 25 µL alkaline lysis buffer. Then, eggs were crushed with a fresh pipette tip and DNA was extracted following the HotSHOT protocol (Montero-Pau et al. 2008) using 25 µL of neutralising buffer.

**Mitochondrial and nuclear marker sequencing**

In order to resolve the phylogenetic relationships of the *Daphnia* species found in the LLC sediments we sequenced mitochondrial and nuclear markers previously used in a phylogenetic analysis of the *D. pulex* species complex (Marková et al. 2013; Vergilino et al. 2011).

A ca. 711 bp fragment of the mitochondrial NADH dehydrogenase subunit 5 (*ND5*) was amplified for 15 resting eggs collected from different sediment layers in LLC and, additionally, for individuals from seven populations of European *D. pulex* (Table S1). We used the recently developed primers *ND5newF* 5'-AAA CCT CTA AAB TTC YKA RCT- 3' and *ND5newR* 5'-CAT RTT YAT RTC RGG GGT TGT- 3' and a modified PCR protocol following Dufresne et al. (2011). The PCR mix contained 0.625 U/µl GoTaq Flexi Polymerase (Promega AG, Dübendorf, Switzerland), 1X Colorless GoTaq Flexi Buffer (Promega AG, Dübendorf, Switzerland), 2 mM MgCl₂, 0.2 mM dNTP's, 0.4 µM of each primer (Microsynth AG, Balgach, Switzerland) and 3 µL of DNA extraction in a total reaction volume of 25 µL. The PCR conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 38 cycles of denaturation at 94 °C for 40 s, annealing at 53 °C for 60 s and elongation at 72 °C for 90 s and a final extension step of 15 min at 72 °C.

Further, we amplified a ca. 550 bp fragment of the nuclear Rab GTPase 4 (*Rab4*) gene for 7 LLC resting eggs (Table S2) using primers *F6for* 5'-CGT TTC GAA TTG GCT TAC TGA-3' and *F12rev* 5'-CAT GGT TAT CTG TCT ACG TCT TGA A-3' (Omilian et al. 2008). Each 25 µL PCR reaction contained 0.625 U/µl GoTaq Flexi Polymerase (Promega AG, Dübendorf, Switzerland), 1X Colorless GoTaq Flexi Buffer (Promega AG, Dübendorf, Switzerland), 2 mM MgCl₂, 0.2 mM dNTP’s, 0.2 µM of each primer (Microsynth AG, Balgach, Switzerland) and 3 µL of DNA extraction. The PCR reaction started with 2 min initial denaturation at 94 °C, followed by 40 cycles of denaturation at 94 °C for 60 s, annealing at 53 °C for 60 s, elongation at 72 °C for 90 s and a final extension step of 10 min at 72 °C.

*ND5* and *Rab4* PCR products were checked on a 1.2 % agarose gel, purified using the Wizard® SV Gel and PCR Clean-Up System (Promega AG, Dübendorf, Switzerland) and sequenced in
both directions by a commercial sequencing service (Microsynth AG, Balgach, Switzerland). Sequences were inspected visually and edited in MEGA 5.1 (Tamura et al. 2011).

For the phylogenetic tree reconstructions we used additional sequences of European *D. pulicaria* and members of the *D. pulex* species complex available on GenBank (Table S1 and S2). Sequences of *D. tenebrosa* served as an outgroup in the ND5 phylogeny and European *D. pulex* in the Rab4, respectively. Alignments, nucleotide substitution model selection based on the Bayesian Information Criterion and maximum likelihood (ML) tree reconstruction, were performed in MEGA 5.1 (Tamura et al. 2011). The ND5 phylogeny was inferred using an alignment of 54 sequences with a length of 591 bp and the Hasegawa-Kishino-Yano nucleotide substitution model with gamma distributed rate heterogeneity (HKY+G) was chosen as the best model (Hasegawa et al. 1985). For the Rab4 ML tree we used an alignment of 15 sequences of 486 bp length and the Tamura 3-parameter (T92) nucleotide substitution model (Tamura 1992). The node support in each tree was assessed by 1,000 bootstrap replicates.

**Microsatellite genotyping**

For an assessment of the genetic diversity of the *D. pulicaria* egg bank in LLC, thirteen microsatellite markers, previously shown to be polymorphic in studies on the *Daphnia pulex* species complex (Colbourne et al. 2004; Dufresne et al. 2011; Pantel et al. 2011; Vergilino et al. 2011), were optimised and combined into four multiplex PCR reactions (MP1-4). All reverse primers were pigtailed (Brownstein et al. 1996) and for Dp525altMM and Dp78MM new primers were designed to improve peak quality (Table S3). Forward primers were fluorescently labelled. All primers were purchased from Microsynth AG (Balgach, Switzerland). PCR reactions contained 1.5 µL of extracted DNA, 5.75 µL Multiplex PCR Master Mix (Quiagen, Hilden, Germany), forward and reverse primers at equimolar concentrations (MP1: 0.2 µM Dp183Mark, 0.7 µM Dp502Mark, 0.3 µM Dp512, 0.35 µM Dp525; MP2: 0.25 µM Dp525altMM, 0.25 µM Dp513, 0.5 µM Dp514, 0.25 µM Dp196; MP3: 0.3 µM Dp78MM, 0.25 µM Dp519, 0.4 µM Dp514alt; MP4: 0.3 µM Dp433, 0.6 µM Dp461) and were filled up with PCR-grade water to a final volume of 11.5 µL. PCR thermal protocols started with an initial denaturation step at 95 °C for 15 min, followed by 33 (MP1, MP2) or 37 (MP3, MP4) cycles of denaturation at 94 °C for 30 s, annealing at 52 °C (MP1, MP4), 54 °C (MP2) or 49 °C (MP3), respectively, for 10 s and elongation at 72 °C for 60 s and were completed with a final elongation step of 30 min at 60 °C.

PCR products were diluted 1:10 (MP1, MP2) and 1:15 (MP3, MP4) in nanopure water, respectively, and 0.5 µL of the diluted PCR product were mixed with 9.25 µL HiDi and 0.2 µL GeneScan500 LIZ size standard (Life Technologies, Carlsbad, CA, USA) and run on an ABI 3130XL Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) for fragment analysis. Binning and scoring of
microsatellite alleles was done in GeneMapper v.4 (Life Technologies, Carlsbad, CA, USA). All samples and alleles were double checked manually. For rare alleles PCRs and genotyping were repeated.

**Population genetics analysis**

Microsatellite data were pooled into eight populations in time frames spanning four to five years, based on available information on pelagial *D. pulicaria* peak abundances (Einsle 1987; Stich & Maier 2007). Standard population genetics parameters and diversity indexes were calculated for each population and compared using GenClone 2.0 (Arnaud-Haond & Belkhir 2007), Arlequin 3.5 (Excoffier & Lischer 2010) and Genetix 4.05 (Belkhir *et al.* 1996-2004). Number of multi-locus genotypes (MLGs), genotypic richness (R), number of alleles, unbiased heterozygosity (unbiased H_e) and the probability that repeated MLGs are the product of distinct sexual events (Psex) were calculated in GenClone 2.0 (Arnaud-Haond & Belkhir 2007). In order to compare populations of different sample size, we also computed genotypic richness, unbiased heterozygosity and number of alleles for the minimum sample size (N=19) running 4,000 permutations in GenClone 2.0 (Arnaud-Haond & Belkhir 2007). Allele frequencies, linkage disequilibrium (LD; 16,000 permutations) and an exact test for Hardy-Weinberg equilibrium (1,000,000 steps in the Markov chain and 100,000 dememorisation steps) were calculated with Arlequin 3.5 (Excoffier & Lischer 2010) and FIS values and 95 % confidence intervals (CIs) were estimated in Genetix 4.05 using 10,000 bootstrap replicates (Belkhir *et al.* 1996-2004). A robust estimate of selfing rates (s) and 95 % CIs were calculated with RMES and selfing rate based FIS values and 95 % CIs were calculated according to the formula $F_{IS} = (s/2-s)$ (David *et al.* 2007; Kopp *et al.* 2012). FIS values and selfing rates were also calculated for a separate dataset without repeated MLGs, to avoid a potential bias introduced by the inclusion of putative obligate parthenogenetic genotypes. Rarefacted allelic richness was estimated with the R-package *hierfstat* (Goudet 2005). In order to assess the presence of distinct clusters and accumulation of genetic variation over time, a discriminant analysis of principal components (DAPC) using the defined populations as cluster priors was performed using the *adegenet* package (Jombart 2008) in R version 2.15.3. (R Core Team 2013). We used the implemented *optim.a.score* function to select the optimum number of PCA axis and retained 10 axis explaining a total of 91.5 % of the variation and 7 discriminant functions. A factorial correspondence analysis (FCA) implemented in Genetix 4.05 (Belkhir *et al.* 1996-2004) was performed to visualize the development of genetic diversity since the first invasion of this *Daphnia* species. Six rare genotypes from different populations with a strong impact on the FCA were removed from this analysis as recommended (Jombart *et al.* 2009).
Hatching experiment

For the assessment of hatching success of resting eggs over time and the functionality of the resting egg bank, we exposed a total of 341 ephippia from three Markelfinger Winkel cores to two main hatching cues, light and increased temperature. Ephippia were incubated singly in filtered lake water (0.45 µm filter, Sartorius Stedim AG, Switzerland) in 48-well plates and exposed to 20 °C and a light-dark cycle of 16:8 h. Hatching was assessed every other day over a minimum period of two weeks and hatchlings were transferred individually to 250 mL jars and kept in culture. Hatching success was calculated as hatchlings per total number of resting eggs and as hatchlings per total number of quality 1 eggs. After the first round of clonal reproduction, hatchlings as well as hatchlings that died before reproduction were stored in 95 % EtOH p.a. (Merck KGaA, Darmstadt, Germany) until analysis. DNA isolated from ethanol samples and from all non-hatched eggs was included in the microsatellite analyses (see above). Confidence intervals (95 %) for hatching success were calculated using the binom package in in R version 2.15.3 (R Core Team 2013) using Wilson's method.

Results

Age model and ephippia density

The obtained $^{137}$Cs and $^{210}$Pb age models for Markelfinger Winkel and Gnadensee sediment cores revealed very similar results. Due to the distinct $^{137}$Cs peak in 1986 (Chernobyl nuclear accident), the $^{137}$Cs models give a better resolution for more recent time periods and were therefore used for dating of the sediment layers. We extracted a total number of 1,407 ephippia from six sediment cores with the first ephippia appearing between end of 1973 and 1975 (Figure 2), which is consistent with the first appearance of *D. pulicaria* in LLC reported by Einsle in 1974 (Einsle 1987). The patterns of ephippia density were comparable between cores and coring locations and we found that they are in accordance with reported abundance peaks of *D. pulicaria* (Einsle 1987; Stich & Maier 2007) in the pelagial of LLC (Figure 2).

Phylogenetic analysis of mitochondrial and nuclear sequence data

In total we obtained 15 sequences of 711 bp of the *ND5* gene from the LLC resting eggs in this study and combined them with three additional sequences from LLC hatchlings that were simultaneously sequenced by Marková et al. (2013). The phylogenetic analysis revealed three distinct *ND5* haplotypes (LLC1, LLC2 and LLC3) in the LLC egg bank (Table S1). LLC1 and LLC2 are distinguished from one another by a single nucleotide substitution, whereas each of these two haplotypes is distinguished from LLC3 by two and one nucleotide substitutions, respectively. LLC1 represents a widely distributed haplotype in European lowland lakes and ponds and in addition we revealed that it was very recently introduced also into Lake Greifensee (Switzerland) (Möst, unpublished data).
Chapter 2

Human-facilitated invasion

Figure 2: Ephippia density in the sediments of Gnadensee and Markelfinger Winkel. Data are pooled from all six aligned cores and presented as the mean ephippia density of non-overlapping windows with size=3 and size=4, respectively, in case of the most recent data point. Black bars indicate periods of abundance peaks of European D. pulicaria reported in the literature.

The most common haplotype LLC2 was identified in 11 samples, LLC1 in seven (two of which were siblings) and LLC3 only in one sample, respectively. In isolates from the period 1974 - 1981, only haplotype LLC2 was detected. The phylogenetic reconstruction of the ML tree (Figure 3) revealed that all ND5 haplotypes found in LLC clustered with the European D. pulicaria haplotypes. Moreover LLC haplotypes were identical or closely related to European D. pulicaria lowland populations from the Czech Republic, Switzerland, Albania, UK, Poland, Germany and the High Tatra Mountains (HTM) populations, but distinct from alpine populations in Switzerland, Austria, Northern Italy and Pyrenees (see also Marková et al. 2013).

Altogether two haplotypes (LLC1 and LLC2) for the Rab4 gene, differing only by a single nucleotide substitution, were obtained. Combining our Rab4 data with sequences from Genbank (Table S2) resulted in a dataset containing 10 polymorphic sites of which 8 were phylogenetically informative. ML phylogenetic analysis of this data set resulted in a well-resolved tree (-log likelihood = -702.84), placing the LLC haplotypes into the European D. pulicaria clade (Figure 4).
Figure 3: ML phylogenetic tree of the mitochondrial ND5 gene. Numbers on major branches represent bootstrap values for 1,000 replications. The scale bar indicates the number of substitutions per site.
Five of seven LLC individuals were homozygous for the LLC1 haplotype and two heterozygous individuals carried the LLC1 as well as the LLC2 haplotype. The LLC1 haplotype is identical to a haplotype found in lowland populations in the Czech Republic (Malá Kuš pond, Chmelnice pond, Chabařovice Lake) and in the UK (King George Reservoir), while the LLC2 haplotype is one of the most widely distributed Rab4 haplotypes in Europe (Table 52) (Marková et al. 2013).

![Figure 4: ML phylogenetic tree of the nuclear Rab4 gene. Numbers on major branches represent bootstrap values for 1,000 replications. The scale bar indicates the number of substitutions per site.](image)

**Microsatellite genotyping and population genetics analysis**

After quality filtering and double checking we retained 519 reliable genotypes, which were used in subsequent statistical analyses. In total, 37 alleles were detected at thirteen microsatellite loci. The average number of alleles per locus was 2.85 with a maximum of eight alleles at locus Dp514alt (Figure 5). Eleven loci were polymorphic, however loci Dp513 and Dp514 were monomorphic in the LLC egg bank, although they are known to be polymorphic in other European D. pulicaria populations (Dufresne et al. 2011; Vergilino et al. 2011, Marková unpublished data). Altogether, 424 multi-locus genotypes (MLGs) were resolved for the full dataset with all populations pooled, and a total of 457 MLGs were detected when populations were analysed singly, ranging from 16 MLGs in population 74-78 to 107 MLGs in population 83-86 (Table 1).
Table 1. Summary table of population genetics parameter calculated for LLC populations.

<table>
<thead>
<tr>
<th>Population</th>
<th>Time span</th>
<th>N</th>
<th>MLGs</th>
<th>R</th>
<th>R*</th>
<th>N alleles (SE)</th>
<th>Ar (SD)</th>
<th>unbiased H_e (SE)</th>
<th>F_s (95% CI) £</th>
<th>F_s (95% CI) £</th>
<th>s (95% CI) §</th>
<th>s (95% CI) §</th>
</tr>
</thead>
<tbody>
<tr>
<td>07-11</td>
<td>2006.8-2011.7</td>
<td>43</td>
<td>43</td>
<td>1.00</td>
<td>1.00</td>
<td>31</td>
<td>2.28 (0.95)</td>
<td>0.357</td>
<td>0.017 (-0.079, 0.09)</td>
<td>0.017 (-0.079, 0.09)</td>
<td>0 (0, 0.216)</td>
<td>0 (0, 0.216)</td>
</tr>
<tr>
<td>02-06</td>
<td>2001.9-2006.8</td>
<td>42</td>
<td>41</td>
<td>0.98</td>
<td>0.99</td>
<td>29</td>
<td>2.21 (0.97)</td>
<td>0.345</td>
<td>-0.014 (-0.12, 0.066)</td>
<td>-0.009 (-0.114, 0.072)</td>
<td>0.001 (0, 0.203)</td>
<td>0.044 (0, 0.202)</td>
</tr>
<tr>
<td>97-01</td>
<td>1997-2001.9</td>
<td>24</td>
<td>24</td>
<td>1.00</td>
<td>1.00</td>
<td>29</td>
<td>2.21 (0.98)</td>
<td>0.342</td>
<td>0.159 (-0.014, 0.289)</td>
<td>0.159 (0, 0.398)</td>
<td>0.183 (0, 0.398)</td>
<td>0.183 (0, 0.398)</td>
</tr>
<tr>
<td>92-96</td>
<td>1992.1-1997</td>
<td>87</td>
<td>87</td>
<td>1.00</td>
<td>1.00</td>
<td>29</td>
<td>2.22 (0.99)</td>
<td>0.339</td>
<td>0.071 (0.003, 0.129)</td>
<td>0.071 (0.003, 0.131)</td>
<td>0.08 (0, 0.21)</td>
<td>0.080 (0, 0.210)</td>
</tr>
<tr>
<td>87-91</td>
<td>1987.2-1992.1</td>
<td>77</td>
<td>74</td>
<td>0.96</td>
<td>0.99</td>
<td>29</td>
<td>2.17 (0.97)</td>
<td>0.309</td>
<td>-0.038 (-0.111, 0.023)</td>
<td>-0.033 (-0.110, 0.03)</td>
<td>0 (0, 0.118)</td>
<td>0 (0, 0.133)</td>
</tr>
<tr>
<td>83-86</td>
<td>1982.6-1987.2</td>
<td>135</td>
<td>107</td>
<td>0.79</td>
<td>0.88</td>
<td>32</td>
<td>2.22 (0.92)</td>
<td>0.312</td>
<td>0.207 (0.128, 0.28)</td>
<td>0.070 (-0.003, 0.136)</td>
<td>0.501 (0.435, 0.556)</td>
<td>0.228 (0.113, 0.331)</td>
</tr>
<tr>
<td>79-82</td>
<td>1978.8-1982.6</td>
<td>92</td>
<td>65</td>
<td>0.70</td>
<td>0.80</td>
<td>33</td>
<td>2.16 (0.93)</td>
<td>0.266</td>
<td>0.241 (0.147, 0.325)</td>
<td>0.073 (-0.016, 0.152)</td>
<td>0.567 (0.492, 0.627)</td>
<td>0.240 (0.091, 0.363)</td>
</tr>
<tr>
<td>74-78</td>
<td>1974-1978.8</td>
<td>19</td>
<td>16</td>
<td>0.83</td>
<td>0.83</td>
<td>19</td>
<td>1.46 (0.52)</td>
<td>0.197</td>
<td>0.141 (-0.211, 0.41)</td>
<td>0.147 (-0.205, 0.424)</td>
<td>0.52 (0.277, 0.658)</td>
<td>0.466 (0.107, 0.645)</td>
</tr>
</tbody>
</table>

N...Number of genotyped resting eggs; MLGs...Multi-locus genotypes; R...genotypic richness, R*...genotypic richness calculated from MLG estimates corrected for minimum sample size (19); N alleles...Number of alleles; Ar...rarefacted allelic richness; unbiased H_e...unbiased heterozygosity; s...selfing rate

§...values corrected for minimum samples size (19) and their standard errors (SE) calculated using 4,000 permutations in GenClone 2.0.
£...values calculated without repeated MLGs
Overall, an increase in genetic and genotypic diversity was detected over time (Table 1). We found a sharp increase in number of alleles and allelic richness between the first population (74-78) and the subsequent populations (Table 1). Strikingly, we never observed more than two alleles per locus in the founding population. A number of 17 new alleles appeared at low frequencies in populations 79-82 and 83-86 of which six alleles disappeared again in the following populations, 10 alleles established in the egg bank and were found in each subsequent population and one allele was only reencountered in 07-11 (Figure 5). In the most recent population 07-11, one additional new allele was observed.

Figure 5: Occurrence of alleles found at 13 microsatellite loci through time. The numbers on the x-axis indicate allele length and labels indicate loci. Circles are drawn at sizes relative to allele frequencies.

Genetic diversity measured as unbiased expected heterozygosity increased by ca. 45 % to a maximum level of 0.36 with a steep increase in early populations that subsequently levelled off with time (Table 1). Considerable departures from Hardy-Weinberg equilibrium and linkage disequilibrium were detected in populations 79-82 and 83-86. This was also reflected by significantly increased $F_{IS}$ values in these populations and marginally but significantly increased $F_{IS}$ values in population 92-96 for the complete dataset. However, $F_{IS}$ values for the dataset without repeated MLGs (457 genotypes) were not significantly different from zero for populations 79-82 and 83-86 (Table 1).
Figure 6: Results of a discriminant analysis of principal components (DAPC) for temporally consecutive *D. pulicaria* populations in LLC. The upper panel depicts a scatter plot using discriminant function 1 and discriminant function 2. Populations are labelled and depicted with different colours and symbols. Inertia ellipses (67%) and a minimum spanning tree are also shown. The lower panel presents the density distribution on discriminant function 1 for each population.

Moreover, considerably high and significant selfing rates were observed in the three oldest populations 74-78, 79-82 and 83-86. Selfing rates calculated for the dataset corrected for repeated
MLGs were lower but remained significant for the same three populations (Table 1). A comparison of $F_{st}$ values with $F_{is}$ values calculated from selfing rates revealed consistent results and did not suggest the presence of null alleles (data not shown).

Identical MLGs and consequently reduced genotypic richness were found in five of the eight populations (Table 1). Notably, these observations could mainly be attributed to the multiple occurrence of a single MLG that was completely homozygous over all loci, occurred already in the founding population 74-78 (2 occurrences), and reached peak abundances in the consecutive populations 79-82 (20 occurrences) and 83-86 (22 occurrences) with very low $P_{sex}$ values ($P_{sex,79-82} < 10^{-57}$ and $P_{sex,83-86} < 10^{-85}$). Other identical MLGs were found only occasionally and at low copy numbers (maximally four copies per MLG) and often represented siblings from the same ephippium. Moreover, most of the LLC populations showed no significant $P_{sex}$ values ($P_{sex} > 0.01$), suggesting that ephippia are the result of sexual reproduction in LLC.

A discriminant analysis of principal components (DAPC) revealed temporal accumulation of genetic changes but no clear separation into distinct populations (Figure 6). Consecutive populations showed a strong overlap, which is best illustrated by their density distributions on the first discriminant function axis (Figure 6). The first two axis of a factorial correspondence analysis (FCA) explained 31.7 % of the genetic variation and reflect an increase in genetic variation, in particular during the early stages (Figure S1).

**Hatching experiment**

Throughout the hatching experiment we observed a total of 86 hatchlings from 372 exposed eggs. Hatching success was higher (> 60 %) for the more recent populations, in particular for population 97-01 (> 80 %). We also found a clear decrease in hatching success with population age (Figure 7). No hatching was observed in population 74-78 and the single hatchling observed in population 79-82 was infertile. Interestingly, opposing trends were observed for hatching success and selfing rates (Figure 7).
**Discussion**

Our analysis of the resting eggs in the LLC sediments clearly shows that a *Daphnia* from the *D. pulex* species complex has invaded LLC around 1974 during peak eutrophication and has subsequently established a population and a functional resting egg bank (Figure 1 and 2). The oldest ephippium found in the sediment cores was dated back to 1973/1974 and this finding is in consistence with the first note of *D. pulicaria* occurrence in the LLC pelagial in 1974 (Einsle 1987). Furthermore, population peaks reported by Einsle (1987) and Stich and Maier (2007) are reflected by the pattern of ephippia densities observed in the sediments (Figure 2). Altogether this suggests that our age model reconstruction is very accurate and that the dynamics of the pelagial population is well represented by the egg bank.

The phylogeny of the *D. pulex* species complex, comprising seven species and up to ten distinct mitochondrial lineages, is complicated and influenced by hybridisation, introgression and polyploidisation (Colbourne et al. 1998; Weider et al. 1999; Vergilino et al. 2009; Vergilino et al. 2011; Marková et al. 2013). Recent studies focusing on Europe have shown that individuals described as *D. pulicaria* are actually belonging to two different taxa, Nearctic *D. pulicaria*, European *D.
**pulicaria** and their hybrids (Marková et al. 2007; Dufresne et al. 2011; Marková et al. 2013). Moreover, a clone of the North American *D. pulex* species complex, identical to an asexual clone described by Mergeay et al. (2006) as an invasive hybrid between American *D. pulex* and American *D. pulicaria* and widely distributed throughout Africa, has recently appeared also in Sardinia and northern Italy (Fadda et al. 2011; Vergilino et al. 2011; Marková et al. 2013). Our phylogenetic analyses of a nuclear and a mitochondrial gene unequivocally placed the *D. pulicaria* isolates from LLC within the European *D. pulicaria* (EPC) clade, which is clearly distinct from the Neartic *D. pulicaria* (Figure 3 and 4, Table S1 and S2). Additional *Rab4* and *LDH-A* analyses by Marková et al. (2013) on LLC hatchlings affirm our finding and put them into a wider European phylogenetic context (see Figure 2 and Figure 3 in Marková et al. 2013). Both *Rab4* haplotypes (LLC1 and LLC2) were identical to many other EPC haplotypes and clustered into a distinct EPC clade (see Figure 2 in Marková et al. 2013). Moreover, the *LDH-A* phylogeny was consistent with previous findings and clustered the LLC haplotype into the EPC clade as well (see Figure 2 in Marková et al. 2013). In conclusion, our results revealed that Lower Lake Constance has been colonised by European *D. pulicaria* and not by other members of the *Daphnia pulex* complex.

European *D. pulicaria* inhabit strongly contrasting habitats, i.e. eutrophic lowland lakes and ponds as well as oligotrophic high-altitude lakes (Hrbáček 1959, 1977; Flößner 2000; Marková et al. 2007; Dufresne et al. 2011). *ND5, Rab4* and *LDH-A* data strongly suggest that LLC has been invaded by populations native to eutrophic lowland carp ponds. Haplotypes that have been found during the initial phase of the LLC invasion are widely distributed in such lowland ponds and lakes in the Czech Republic and are distinct from haplotypes found in high-altitude habitats in the Alps and Pyrenees (Marková et al. 2013). The fact that the *ND5* haplotype LLC1 is also found in an alpine lake in the High Tatra Mountains (HTM) is not necessarily a contradicting finding. As Dufresne et al. (2011) have shown before, the HTM have recently been colonised from different glacial refugia and therefore share some *ND5* haplotypes with lowland populations (see Figure 4 in Dufresne et al. 2011). Based on currently available data, the remaining two *ND5* haplotypes (LLC2 and LLC3) are unique to LLC and the fact that they differ by only one nucleotide substitution suggests that they may have evolved locally.

Changes in resource availability, e.g. eutrophication, can increase the invasibility of communities (Davis et al. 2000; Davis & Pelsor 2001) and thus facilitate invasions (Chase & Knight 2006). Eutrophication has been shown already to play a central role in successful invasions of *Daphnia galeata* into peri-alpine lakes, among them Upper Lake Constance (Brede et al. 2009; Rellstab et al. 2011; Spaak et al. 2012). Overall, our data suggest that an invasion of LLC by individuals from populations adapted to meso- and eutrophic lowland habitats, probably located in Central
Europe, has been facilitated by human-caused eutrophication of LLC as previously suggested (Einsle 1987; Flößner 2000).

We can only speculate about possible vectors for the invasion of European *D. pulicaria*. Lake Constance and lakes harbouring potential source populations are situated within the Mediterranean/Black Sea flyway for migratory birds (Boere 2006), rendering water fowl a likely vector. However, human mediated transport of propagules, e.g. via fishing gear, boats or fish stocking, cannot be ruled out.

The initial phases of invasions and the development of genetic diversity are of particular interest for understanding the invasion process and the conditions leading to a successful establishment (Roman & Darling 2007). Especially for aquatic species in large water bodies it is likely that these initial stages are overlooked and not completely captured or the invasion has already taken place at a time when appropriate genetic methods have not yet been available. We therefore used the resting egg bank of European *D. pulicaria* as a proxy to reconstruct the long-term evolutionary dynamic of invasion and establishment of the species in LLC from the early 70s until 2011. The pattern of genetic diversity we report here is consistent with a scenario of multiple introductions (Kolbe *et al.* 2004; Kelly *et al.* 2006; Roman & Darling 2007) and clearly different from a scenario of an invasion of a single asexual genotype, as described for another member of the *D. pulex* species complex in African lakes (Mergeay *et al.* 2006). While the diversity of the first population is extremely low with no more than two alleles per locus and could therefore in principal represent an invasion by a single clone, all measures of genetic diversity and richness show a steep increase during the first 12 years (Table 1, Figure 5, Figure S1). In the course of time, genetic diversity, measured as expected heterozygosity and allelic richness, reached levels that lie within a range that is typically encountered in populations of European *D. pulicaria* (Dufresne *et al.* 2011). Moreover, the appearance of new microsatellite alleles especially in populations 79-82 and 83-86 and the first notice of the *Rab4* LLC2 haplotype towards the end of the 1980s are indicative for additional introductions during that time. We also noticed one new allele, suggesting that propagules are still transported to Lower Lake Constance. Multiple introductions and a consequent increase in genetic diversity can be decisive for successful establishment and the potential for range expansion of an introduced species (Roman & Darling 2007). An alternative explanation for our data could be a single introduction already comprising all the genetic diversity with only a small subset of clones reaching high abundances and producing resting eggs. Such a scenario would, however, need to invoke that several clones persist clonally in the pelagial over more than a decade until they finally produce ephippia or males. Moreover, studies on the abundance of European *D. pulicaria* revealed high peaks followed by long periods of virtual absence from the pelagial (Einsle 1987; Stich & Maier 2007), a
pattern ascribed to high fish predation pressure on this large-bodied species (Stich & Maier 2007). Consequently, the European *D. pulicaria* population in LLC relies heavily on its egg bank and long-term existence of clones in the pelagial seems unlikely. A scenario of multiple introductions appears thus to be the most parsimonious explanation.

A further important conclusion is apparent from our analysis of the development of genetic structure over time (Figure 6). The multiple invasions have not replaced each other as one could expect given the strongly fluctuating pelagial abundance pattern (Einsle 1987; Stich & Maier 2007). Instead, the genetic changes of the population in LLC evolve gradually through time with a strong overlap of consecutive populations, a pattern that is readily explained by the buffer effect of the egg bank (Figure 6).

The fact that some alleles appear in populations 79-82 and 83-86 but apparently did not manage to establish and that hardly any new alleles accrue after the first three populations (Figure 5) is also well in accordance with the monopolisation hypothesis (De Meester et al. 2002). This observation coincides with a filling up of the egg bank (Figure 2) and a flattening of the increase of genetic diversity (Table 1). According to the monopolisation hypothesis, such a pattern is explained by rapid local adaptation and population growth during the initial phase of colonization. This results in strong priority effects through the monopolisation of resources and, together with the build-up of a large resting egg bank serving as a buffer, decreases the establishment success of new invading genotypes (De Meester et al. 2002). Although we cannot directly show local adaptation with our data, our results are in line with the monopolisation hypothesis. The selective advantage of the locally adapted population and the decrease in new alleles establishing in LLC may have been also reinforced by the on-going re-oligotrophication process.

A further characteristic that one would expect for an invasion founded by a small number of genotypes of a cyclical parthenogenetic species is also reflected by the population genetics analysis of the egg bank. We identified significant selfing rates during the initial stages, also remaining significant after the exclusion of repeated MLGs, showing a decline over time. As long as the number of genotypes in the lake and in the egg bank is low it is likely that the pelagial population is dominated by clones of only few different genotypes, increasing the chance of selfing (i.e. intraclass mating) and biparental inbreeding during sexual reproduction. With the arrival of new genotypes and the filling of the egg bank the number of different genotypes available for mating in the pelagial is expected to increase and selfing and inbreeding become more unlikely. This hypothesis is consistent with the observed decrease of selfing rates with increasing genetic diversity and the accumulation of resting eggs in LLC.
Intriguingly, we observed a dominance of a single homozygous MLG in populations 79-82 and 83-86, resulting in significant $F_{is}$ values and strong LD in these populations. One possible explanation for such an observation could be obligate parthenogenesis. Indeed, obligate parthenogenesis is known for members of the *D. pulex* species complex (Colbourne et al. 1998). Recently, Dufresne et al. (2011) suggested obligate parthenogenesis as reproductive mode for European *D. pulex* in the HTM and a mix of obligate parthenogenesis and sexual reproduction for lowland populations. Assuming obligate apomictic parthenogenesis we would expect a high level of heterozygosity at different loci due to the accumulation of mutations (e.g. Hebert 1981) and therefore a homozygous obligate apomictic parthenogenetic genotype seems likely only if it has recently emerged from a highly homozygous individual. High levels of homozygosity could be possibly explained by obligate aapomictic parthenogenesis (Simon et al. 2003). It has been shown for *D. pulex* that, mechanistically, parthenogenetic reproduction represents an atypical automixis via abortive meiosis (Hiruta et al. 2010) that, however, results in a genetic patterns similar or equivalent to apomictic parthenogenesis. Interestingly, transitions to obligate parthenogenesis have been reported in European *D. pulex* (Marková et al. 2007; Dufresne et al. 2011) inhabiting alpine lakes in the HTM (Dufresne et al. 2011). However in that case obligate parthenogenesis was associated with increased heterozygosity and most probably originated from hybridisation between divergent mtDNA linea (European *D. pulex* and North American *D. pulex*, see Marková et al. 2007, Dufresne et al. 2011; Marková et al. 2013). An alternative explanation for the presence of repeated homozygous MLGs in LLC could be strong inbreeding (i.e. biparental inbreeding and selfing). For LLC we would need to invoke the production of highly homozygous individuals due to inbreeding or colonisation by already inbred individuals during the initial phase of the invasion followed by a dominance of this genotype in the pelagic population during certain times in the periods 79-82 and 83-86 with inbreeding that maintains homozygosity. The fact that the homozygous genotype disappeared from the egg bank with increasing genetic diversity and decreasing selfing rates supports this hypothesis.

Our hatching experiment revealed that the egg bank is functional as we observed fertile hatchlings up to 29 years back in time. Although the observed age for successful hatching is not exceptional for *Daphnia* resting egg banks it is at the lower limit compared to other studies (Weider et al. 1997; Cáceres 1998; Hairston et al. 1999a,b; Keller & Spaak 2004; Hembre & Peterson 2013). We identified an intriguing coincidence of decreasing selfing rates with increasing hatching success (Figure 7). Despite of the fact that inbreeding depression is known for *Daphnia* spp. (De Meester 1993; Deng 1997; Haag et al. 2002; Cáceres et al. 2009) and could potentially have contributed to the reduced hatching success in the oldest populations, our present data do not allow to disentangle effects of inbreeding and senescence on hatching success.
In conclusion, by analysing the LLC resting egg bank we were able to reconstruct the genetic processes associated with the invasion of a key planktonic grazer. Our data suggest the following scenario: European *D. pulicaria*, most likely originating from lowland populations in Central Europe and pre-adapted to eutrophic conditions, invaded LLC around 1974 and successfully established a population and a resting egg bank. This process was facilitated by the human-caused eutrophication of LLC. The founder population comprised very few genotypes, possibly only one, and further introductions contributed to an increase of genetic diversity and evolutionary potential (Sakai *et al.* 2001; Kolbe *et al.* 2004; Frankham 2005a; Kelly *et al.* 2006; Roman & Darling 2007). The ability to reproduce asexually via either facultative or obligate parthenogenesis or a mix of both and to produce resting eggs has allowed the species to overcome negative consequences of a small founding population during the initial phases of the invasion (e.g. inbreeding depression, Allee effects, high extinction risk) and to establish successfully (Sakai *et al.* 2001; Roman & Darling 2007).

In the course of time, the European *D. pulicaria* population in LLC monopolised the available resources and the chance of establishment for further immigrants, and therefore the increase of genetic diversity, ceased (De Meester *et al.* 2002). Interestingly, European *D. pulicaria* with a mitochondrial ND5 haplotype identical to the one found in LLC (Figure 3, Table S1) has been detected for the first time in the pelagial of the nearby Lake Greifensee in winter 2012. Additional work is now required to monitor whether this invader is able to successfully establish a population in Lake Greifensee and whether it expands into other surrounding lakes.

The system we describe here has the potential to serve as an excellent model to study invasions and adaptation of populations to changing environmental conditions, e.g. by applying paleogenomics approaches (Orsini *et al.* 2013), because this study describes the population history, LLC has undergone a rapid environmental change in recent years that is well documented, and a reference genome for the *D. pulex* complex (Colbourne *et al.* 2011) is already available. We are convinced that our findings will contribute significantly to the understanding of genetic processes associated with successful invasions. Moreover, our results highlight the importance of considering human-caused environmental changes in creating opportunities for biological invasions.

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References


Boere GC (2006) Waterbirds around the world a global overview of the conservation, management and research of the world’s waterbird flyways Stationery Office, Edinburgh, UK.


## Supplementary Information

Table S1. Sample information for ND5 sequences. Sequences for 15 resting eggs from LLC and eight individuals from European populations were obtained in this study. Additional sequences for four hatchlings from LLC were sequenced by Marková et al. (2013) and the remaining sequences used for the phylogenetic reconstruction were either provided by Marková et al. (2013) or were available from GenBank.

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**Table S2.** Sample information for *Rab4* sequences. Sequences for six resting eggs were obtained in this study. One additional sequence for a hatching from LLC was sequenced by Marková et al. (2013) and the remaining sequences used in our analysis were available from GenBank.

<table>
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<th>Haplotype</th>
<th>Sample(s)</th>
<th>Location/Lake (Age)</th>
<th>Country</th>
<th>Latitude N</th>
<th>Longitude E</th>
<th>Altitude (m a.s.l.)</th>
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<th>Accession Number</th>
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Table S3. Summary information on microsatellite markers and primers used.

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<td>Dp502Mark</td>
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Figure S1: The first two axes of a factorial correspondence analysis (FCA) showing the increase of genotypic diversity of European D. pulicaria in LLC over time. Temporally consecutive populations are indicated by different colours and symbols. The percentage of variation explained by each axis is shown in parentheses.
Chapter 3: Environmental organic contaminants influence hatching from *Daphnia* resting eggs and hatchling survival

In submission to *Aquatic Toxicology*

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\(\text{a}\) A. C. Chiaia-Hernandez and M. Möst contributed equally to this work

Abstract

The occurrence and fate of contaminants in surfaces waters are of great environmental concern and pose a threat to aquatic animals, ecosystems and water safety. Risk assessment of organic contaminants toward aquatic organism is difficult and the study of joint toxicity is even more challenging, since most of the organic contaminants are present in different concentrations and the complex life cycles of many aquatic species within different compartments complicate their study. A mixtures of organic contaminants found in sediments of Lake Greifensee were selected to study their effect on hatching ability and hatchling mortality using resting eggs from a *Daphnia* species complex inhabiting this lake. In this work, the effect of mixture toxicity on hatching success of resting eggs and hatchling mortality was assessed, finding a significant increase in hatching success and hatchling mortality for resting eggs exposed to organic contaminants. Increasing hatching success has not yet been reported from ecotoxicological studies and adds a novel aspect that requires consideration in risk assessments. In this study, mechanistic explanations for our results are discussed, as well as the potential implications on the ecology and evolution of aquatic species that rely on a resting egg bank. Furthermore, our results highlight the need of further studies assessing the effects of organic contaminants on benthic-pelagic coupling and aquatic ecosystems.

**Key Words:** Mixture toxicity, micropollutants, resting egg bank, diapause, benthic-pelagic coupling, ephippia, water flea
Introduction

Among the most important planktonic grazers in freshwater pelagic food webs are species of the genus *Daphnia* (Crustacea: Anomopoda; water fleas). *Daphnia* species constitute a major food source for fish and invertebrates, are important planktonic grazers, and therefore are attributed as keystone species in lentic ecosystems (Lampert 2011). *Daphnia* spp. have been established as important model organisms in aquatic toxicology (Martins et al. 2007; Altshuler et al. 2011; Colbourne et al. 2011; Lampert 2011) and have recently been adopted as one of 13 model organisms for biomedical research by the National Institutes of Health (NIH 2013). Most toxicological studies are conducted with the large-bodied *D. magna* (e.g. OECD 2004; Kretschmann et al. 2011; OECD 2012; Jeon et al. 2013), a species known to preferentially inhabit ponds. Less attention has so far been paid to other species such as the members of the *D. longispina* species complex despite their significance for large lakes ecosystems and importance for drinking water reservoirs, e.g. Lake Zurich and Lake Constance (Lampert 2011; Spaak et al. 2012).

Most *Daphnia* species reproduce clonally (parthenogenetic cycle) during favourable conditions but switch to sexual reproduction (sexual cycle) when environmental conditions are not ideal, triggered by e.g. changes in food level, crowding and photoperiod (Zaffagnini 1987; Ebert 2005; Lampert 2011). During sexual reproduction, *Daphnia* produce dormant eggs, enclosed in a protective case called ephippium. Ephippia can float on the lake surface and may be transported by wind (Vanschoenwinkel et al. 2008), waterfowl (Figueroa & Green 2002; Figueroa et al. 2003), insects (Van de Meutter et al. 2008) or human activities (Havel & Shurin 2004; Stasko et al. 2012) to other water bodies. A portion of the resting eggs sinks to the bottom of the lake where they contribute to the build-up of a so-called resting egg bank (Brendonck & De Meester 2003).

Resting egg banks are not only known for *Daphnia* but for many aquatic organisms and play a crucial role for their ecology and evolution (for comprehensive reviews on egg banks see Hairston & Kearns 2002; Brendonck & De Meester 2003; Gyllstrom & Hansson 2004). Parts of or even the entire active pelagial population are recruited from the egg bank during each growing season via trans-generational hatching, and the egg bank is in return re-stocked with dormant eggs produced by the active population. This interdependence, known as benthic-pelagic coupling, affects population dynamics and the evolutionary potential of many zooplankton species (Cáceres & Hairston 1998; Gyllstrom & Hansson 2004). First of all, the egg bank allows to escape from detrimental biotic (e.g. Slusarczyk 1995) or abiotic factors (e.g. Hairston & Olds 1987) and reduces thus the extinction risk of local populations. Furthermore, in fluctuating environments, the egg bank helps to maintain the co-existence of species and genotypes via the storage effect (Chesson & Warner 1981; Chesson 1983;
Cáceres 1997). The egg bank allows to preserve and increase genetic diversity (Ellner & Hairston 1994; Hedrick 1995) and thereby the evolutionary potential of species and buffers local populations against the establishment of immigrants (De Meester et al. 2002).

In addition to their important role for the biology of many aquatic species, dormant eggs can also be used to reconstruct the impact of human-caused environmental change (Hairston et al. 1999; Jankowski & Straile 2003; Kerfoot & Weider 2004; Brede et al. 2009; Rellstab et al. 2011). In deeper parts of lakes, dormant eggs do not receive hatching stimuli and remain in the sediment, providing an unbiased archive of past populations. Dormant eggs extracted from the sediments can be hatched for experimental purpose or directly be analysed with molecular genetic methods several decades back in time (Weider et al. 1997; Limburg & Weider 2002; Orsini et al. 2013).

Due to the key role of Daphnia spp. in aquatic food webs, their particular life history features, comprehensive understanding of their distribution and biology, as well as the wealth of toxicological information and genomic tools available (Colbourne et al. 2011), Daphnia is an ideal model system to understand the impact of abiotic factors on lentic ecosystems. For example, Brede et al. (2009) and Rellstab et al. (2011) analysed resting eggs of the Daphnia longispina-galeata species complex from five European lakes showing a correlation of an increase of D. galeata abundance, interspecific hybridisation, and a decrease of D. longispina abundance over time with human-induced changes in total phosphorus levels.

Today, many water bodies in western countries have recovered from anthropogenically induced eutrophication. Nevertheless, pollutants, in particular so called micropollutants, are still introduced into these systems, representing future threats to natural populations of aquatic organisms (Schwarzenbach et al. 2006; Sumpter 2009; Kadokami et al. 2013). Organic contaminants can enter natural waters from different sources, e.g. via waste water treatment plants effluents, urban and industrial sewage, surface runoff, spray drift and leaching from agricultural areas. Depending on their physical-chemical properties, they sorb to the sediment and form an excellent archive of former pollution. This was shown for highly lipophilic compounds like PCBs (Zennegg et al. 2007; Kohler 2008) but recently also for medium polar contaminants such as pesticides, personal care products, biocides and corrosion inhibitors (Chiaia-Hernandez et al. 2013a). Such medium polar contaminants are much more bioavailable for freshwater organisms as opposed to highly lipophilic organic contaminants (Neff 1984).

Organic contaminants from the particulate and interstitial components of sediments as well as from the water column constitute a primary source of exposure for benthic organism and their life cycle stages (Swartz & Lee II 1980; Knezovich & Harrison 1987). Sediments contaminated with
mutagenic substances are known to pose a hazard to indigenous biota including adverse effects such as DNA damage, chromosomal aberrations and cancer (Chen & White 2004). In addition, chemical pollutants in aquatic system have been associated with reproductive impairment (Hayes et al. 2002), limb deformities (Kiesecker 2002) and declines of non-targeted species (Liess & Schulz 1999; Davidson et al. 2002; Liess & Von der Ohe 2005).

Despite of the general importance of egg banks for many planktonic organisms and the known accumulation of contaminants in lake sediments, the number of studies addressing a potential effect of pollutants on the function of egg banks is limited, and in some cases with inconclusive results as summarized by Navis et al. (2013): Angeler et al. (2005) detected a negative impact of a commercial fire retardant on the emergence success of *D. curvirostris*. In addition, Raikow and colleagues showed an effect of ballast tank treatments SeaKleen (menadione) (Raikow et al. 2006) and sodium hypochlorite (Raikow et al. 2007) on the hatching of resting eggs of different planktonic organisms, among them *Daphnia mendotae*. For *Artemia* cysts no effect of the pesticide chlorpyrifos on hatching could be shown (Varo et al. 2006) and studies on the effect of cadmium and zinc revealed contradicting results (Bagshaw et al. 1986; Rafiee et al. 1986; Sarabia et al. 2003; Sarabia et al. 2008). However, a detrimental effect of heavy metal exposure on the hatching success of the marine copepod *Acartia pacifica* was reported (Jiang et al. 2007). Marcial et al. (2005) studied the hatching abilities of the rotifer *Brachionus plicatilis* when exposed to four different pesticides, reporting that sexual reproduction is a more sensitive parameter than asexual reproduction, and that resting egg hatchability is the most sensitive parameter in detecting effects of the pesticide diazinon when resting eggs are exposed during their development (Marcial & Hagiwara 2007). In a recent study on *D. magna*, Navis et al. (2013) found evidence for negative effects of the endocrine disruptor fenoxycarb and the insecticide carbaryl on survival and reproduction of hatchlings, as well as the impact of fenoxycarb on the development of resting eggs and hatching success.

While egg bank and benthic-pelagic coupling may be adversely affected at different levels of the life cycle (Navis et al. 2013), in this work, we focused on the direct effects of toxicants on resting eggs (i.e. hatching success and hatchling mortality). We followed a new approach as we exposed ephippia of a natural population of the *D. longispina* species complex, directly collected from the surface of Lake Greifensee (Switzerland) after production, to a mixture of organic contaminants that were detected in the sediments from the same lake. In our previous work we showed that organic contaminants can bioaccumulate in ephippia of these species in uptake and elimination experiments. Additionally, ephippia internal concentrations in the environment were predicted based on the past and recent contamination of lake sediments, using the equilibrium partitioning model (EqP) (Chiaia et al. 2013b). Therefore, the main objectives of this study were to (i) establish an experimental setup
for ecotoxicological tests using *D. longispina* species complex ephippia and to (ii) test for a potential impact of a mixture of contaminants, found in the sediments of Lake Greifensee and 1,000 fold increased in concentration with respect to the environmental concentrations of each component, to clarify whether an effect can be observed on hatching abilities of resting eggs, and consequently on the ecology and evolution of a species complex that occupies a key role in the ecosystem of most European large lakes.

**Material and Methods**

**Ephippia collection and preparation**

Ephippia produced by the *Daphnia longispina* species complex were collected with nets pulled after a boat from the surface (i.e. within a few days after their production) of Lake Greifensee (N47.353950°/E8.6758917) during peaks of sexual reproduction in spring and fall 2011 and stored in 10 L polyethylene (HD-PE) bottles (Hünersdorff GmbH, Germany). Ephippia were cleaned using sieves of different mesh sizes, rinsed with filtered and double autoclaved lake water and stored in the dark at 4 °C in 500 mL Schott bottles (Schott Duran, Germany) until processing, in order to break diapause. Ephippia were dried in the dark at 4 °C and subsequently pooled together and mixed before the experiment.

Lake water used for storage and exposure experiments was collected from Lake Greifensee, filtered through a glass fiber filter (pore size: 0.45 μm, Sartorius Stedim AG, Switzerland) and double autoclaved at 120 °C for 30 min each time with a Vapoklav 500 (HP Medizintechnik GmbH, Germany).

**Standards and reagents**

Reference standards used in the experiment had a purity of 97 % or greater. Irgarol, triclocarban, benzotriazole, 5-methylbenzotriazole and octocrylene were purchased from Sigma-Aldrich (Steinheim, Germany). Propiconazole, terbutryn and prochloraz were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Triclosan was provided by Ciba (Basel, Switzerland) and tonalide was purchased from LGC Standards (Wesel, Germany).

**Hatching Experiment**

For the exposure experiment, dry ephippia were moisturized and aliquots of 0.15 mL were transferred to 15 mL polypropylene centrifuge tubes (VWR, Dietikon, Switzerland). Assay tubes were randomized and filled with 14 mL of exposure medium or control medium, respectively. For each treatment (exposure and control), 30 tubes were prepared with a total of 60 assays per experiment. The exposure medium consisted of filtered and double autoclaved lake water containing a mixture of 10 analytes with a nominal final concentration between 2 to 800 μg/L. Concentrations were selected
according to the predicted pore water maximum concentration \( (C_{pw,max}) \) based on sediment analyses from Lake Greifensee reported by Chiaia-Hernandez et al. (2013a,b), multiplied by a factor of 1,000. The exposure mixture included pesticides, corrosion inhibitors, biocides, and personal care products. The chemicals were spiked as a mixture in ethanol with a final ethanol content of 0.2 % (v/v). The complete list of analytes with measured concentrations and physicochemical characteristics are reported in Table 1. The control medium contained filtered and double autoclaved lake water with a final ethanol content of 0.2 % (v/v).

Ephippia were pre-incubated (day 0) for four days at 4 °C to allow them to equilibrate with the exposure medium and reach steady state for bioconcentration of contaminants as was determined in previous toxicokinetic experiments (Chiaia-Hernandez et al. 2013b). After pre-incubation, ephippia were transferred to an incubator to stimulate hatching at a temperature of 20 °C and a light:dark cycle of 16:8 (Memmert GmbH & Co. KG, Schwabach, Germany). In the incubator, the assay tubes were randomized and placed at a 10° angle in order to ensure optimum contact between the ephippia and the medium. Assay tubes were randomized daily and examined on day 4, 7, 10, 13 and 15 under a stereo microscope. Simultaneously, exposure and control media were renewed to avoid sorption and degradation of chemicals in the exposure medium due to the experimental setup. Hatchlings were counted, removed from the assay tubes and mortality was recorded. The complete experiment was performed twice during different seasons of the year, i.e. December 2012 and March 2013.

Quality controls were taken during the experiment and included freshly prepared control and exposure media, as well as control and exposure media taken from random assay tubes while exchanging the media. The quality controls were collected on day 0, 4, 7, 10, 13 and 15 in duplicates and analysed for possible cross contamination and stability of the exposure medium. No contamination was found in the quality controls.

The stability of the chemicals during the experimental conditions was explored in independent studies. Photodegradation was studied with the chemical mixture reported in Table 1 with a nominal final concentration of 200 \( \mu \text{g}/\text{L} \) using glass jars and under similar experimental conditions as described above. Samples were taken at the beginning of the experiment (0 h) and every 24 h for six consecutive days in triplicates. The results show a degradation under light exposure of 73 % and 62 % for triclosan and tonalide, respectively, after 72 h. The results are consistent with the reported photolysis of triclosan and tonalide in surface and waste water (Singer et al. 2002; Santiago-Morales et al. 2012). No photolysis was observed for the rest of the compounds as illustrated in Figure S1 and Table 1. Additionally, a separate study was performed to study the sorption of chemicals to polypropylene tubes under dark conditions. The results listed in Table 1
show that most compounds do not significantly sorb to plastic within 72 h but 67 % and 86 % of triclosan and tonalide, respectively, are lost and therefore not bioavailable over the whole time range of the experiment. The fivefold exchange of the medium during the experiment accounted for those losses due to photolysis and sorption, more frequent exchange was not feasible as we aimed to keep disturbance of the hatching process at minimum.

Control and exposure media were directly analysed by transferring 940 μL of medium to 1 mL HPLC vials (BGB Analytics AG, Switzerland) followed by addition of 60 μL of internal standard mix solution and analysed by LC-HRMS as described elsewhere (Chiaia-Hernandez et al. 2013b). In addition, pH (8.2 ± 0.2) was monitored during the experiment. Furthermore, dissolved oxygen was measured for a subset of samples to check for potential differences between control and exposure medium, however, no such differences were detected.
Table 1. Analytes with measured concentrations ($C_{med}$) and physicochemical characteristics used in the exposure experiment. Degradation by light using glass containers and sorption to polyethylene tubes under dark conditions were studied independently under similar experimental conditions.

*Compounds known to have antimicrobial activity, $C_{ipw:max}$ is the maximal (max) predicted pore water concentrations based on analysis of sediment from Lake Greifensee previously reported (Chiaia-Hernandez et al. 2013 a,b). $C_{med}$ is the average concentration at $t=0$ before distribution and exchange of the medium in the assays, $C_{med-D}$ is the average medium concentration during incubation at dark and at 4 °C, $C_{med-L}$ is the concentration of the medium under light exposure and at room temperature.

1Log Dow (Octanol-water distribution coefficient) values were predicted using MarvinSketch 5.11 (ChemAxon http://www.chemaxon.com)


<table>
<thead>
<tr>
<th>Name</th>
<th>CAS #</th>
<th>Compound class</th>
<th>$^a$log Dow at pH 8.2</th>
<th>$C_{ipw:max}$ x1,000 (µg/L)</th>
<th>$C_{med}$ (µg/L)</th>
<th>$C_{med-D}$ (µg/L)</th>
<th>$C_{med-L}$ (µg/L)</th>
<th>Degradation by light at 20°C t=72hrs (%)</th>
<th>Sorption to polyethylene tubes t=72hrs (%)</th>
<th>$^b$EC50 (µg/L)</th>
</tr>
</thead>
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<tr>
<td>Benzotriazole</td>
<td>95-14-7</td>
<td>Corrosion inhibitor</td>
<td>1.2</td>
<td>660</td>
<td>650 ± 60</td>
<td>650 ± 60</td>
<td>630 ± 40</td>
<td>Stable</td>
<td>0</td>
<td>35,000-280,000</td>
</tr>
<tr>
<td>5-Methylbenzotriazole</td>
<td>49636-02-4</td>
<td>Corrosion inhibitor</td>
<td>1.4</td>
<td>510</td>
<td>740 ± 50</td>
<td>650 ± 70</td>
<td>700 ± 70</td>
<td>Stable</td>
<td>0</td>
<td>35,000-280,000</td>
</tr>
<tr>
<td>Irgarol</td>
<td>28159-98-0</td>
<td>Biocide</td>
<td>3.0</td>
<td>210</td>
<td>450 ± 40</td>
<td>360 ± 20</td>
<td>350 ± 30</td>
<td>Stable</td>
<td>7</td>
<td>8100</td>
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<tr>
<td>Octocrylene</td>
<td>6197-30-4</td>
<td>PCP</td>
<td>6.8</td>
<td>2</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>Stable</td>
<td>19</td>
<td>1,000-75,000</td>
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<td>Prochloraz*</td>
<td>67747-09-5</td>
<td>Pesticide</td>
<td>3.6</td>
<td>35</td>
<td>19 ± 1</td>
<td>14 ± 1</td>
<td>16 ± 1</td>
<td>Stable</td>
<td>6</td>
<td>4300</td>
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<tr>
<td>Propiconazole*</td>
<td>60207-90-1</td>
<td>Biocide</td>
<td>4.3</td>
<td>24</td>
<td>19 ± 1</td>
<td>15 ± 1</td>
<td>16 ± 1</td>
<td>Stable</td>
<td>6</td>
<td>2,600-13,000</td>
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<tr>
<td>Terbutryn</td>
<td>886-50-0</td>
<td>Biocide</td>
<td>2.9</td>
<td>28</td>
<td>29 ± 2</td>
<td>23 ± 1</td>
<td>23 ± 2</td>
<td>Stable</td>
<td>7</td>
<td>2,600-7,100</td>
</tr>
<tr>
<td>Tonalide</td>
<td>21145-77-7</td>
<td>PCP</td>
<td>5.0</td>
<td>510</td>
<td>500 ± 100</td>
<td>47 ± 4</td>
<td>13 ± 5</td>
<td>62</td>
<td>86</td>
<td>239-249</td>
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<tr>
<td>Triclocarban*</td>
<td>101-20-2</td>
<td>Biocide</td>
<td>4.9</td>
<td>820</td>
<td>260 ± 40</td>
<td>320 ± 2</td>
<td>300 ± 100</td>
<td>Stable</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Triclosan*</td>
<td>3380-34-5</td>
<td>Biocide</td>
<td>4.4</td>
<td>320</td>
<td>430 ± 330</td>
<td>120 ± 20</td>
<td>73 ± 13</td>
<td>73</td>
<td>67</td>
<td>390-560</td>
</tr>
</tbody>
</table>
Ephippia and egg counting

Ephippia were counted after the experiment in order to correct for differences in the number of ephippia per assay tube. Ephippia were arranged on 8 x 11 cm glass slides with a white background and photographed with a digital camera (Panasonic DMC-FZ50 camera, Japan, with a Leica DC-Vario-Elmarit 1:2.8-3.7/7.4-88.8 ASPH zoom lens, Switzerland). Subsequently, the photographs were used to automatically count ephippia in batch processing mode using CellC 1.2. software (Selinummi et al. 2005). The implemented cluster division algorithm based on cell shape for light microscopy images was used with cluster division set to a value of 1 and with a background correction applied prior to processing. Moreover, two different intensity thresholds (0.4 and 0.25) were employed to represent a range of ephippia for which reliable results in previous tests had been obtained. The difference between the two ephippium count datasets was minor with 2.1 % on average (s.d. 1.1), a maximum difference of 6.1 % and no relevant difference between controls and exposed ephippia. Therefore, only results obtained for intensity threshold 0.25 are reported which yielded a slightly better ephippia count.

In order to calculate the number of eggs for the analysis of hatching success, an average egg content per ephippium was determined by opening a subsample of 100 ephippia and counting the eggs under a stereo microscope. The number of exposed eggs in each assay tube was calculated by multiplying the ephippia count datasets with the average egg content (0.17 eggs/ephippium).

Statistical analysis

The binomial response variables hatching success and mortality were evaluated with a generalized linear model (GLM). In order to account for overdispersion, present in both cases, a quasi-binomial model was employed.

For hatching success the full model with the fixed factors treatment and experiment and their interaction as explanatory variables was first tested. The interaction term was not significant in a partial F-test comparing the full model against a model without interaction. Therefore, the interaction term was removed and a simpler model containing the fixed factors treatment and experiment was used (Table 2). The analysis was performed with datasets based on both ephippia counts and both analyses revealed a similar outcome with no relevant differences.

For mortality, the full model with the fixed factors, treatment and experiment; and their interaction as explanatory variables was tested. The interaction term was highly significant and the full model was kept (Table 3). One extreme outlier was removed from the mortality dataset to improve the model with no relevant effect on the significance of the terms.

Residual analyses conducted for all models revealed no violation of model assumptions.
All models were evaluated using the function \textit{glm} and partial F-tests for the significance of single terms in the models and were performed using the function \textit{drop1} in R version 3.0.1 (R Core Team 2013).

**Results and Discussion**

This study reveals that ecotoxicological assays using resting eggs from the \textit{D. longispina} complex are suited to investigate the impact of organic contaminants found in sediments of large lakes and drinking water reservoirs, on a key species in aquatic ecosystems. Water bodies with large catchment areas can serve as sink for complex mixtures of organic contaminants from different diffuse and point sources. Therefore, the threat that different contaminants pose to the freshwater ecosystems, is now widely recognised and has raised attention in ecotoxicological research (Fent \textit{et al.} 2006; Schwarzenbach \textit{et al.} 2006; Sumpter 2009; Haarstad \textit{et al.} 2012).

Despite the complex mixtures of different organic contaminants found in the environment, the study of mixed toxicity has not been established and studies of joint toxicity with existing natural populations are scarce (Relyea & Hoverman 2006). Additionally, many studies focus either on single substances (Relyea & Hoverman 2006) or use combinations of contaminants and test organisms, that are uncertain or even unlikely to actually occur together in nature (Chapman 2002; Freitas & Rocha 2011). Furthermore, particular life cycle stages and compartments occupied by species during certain phases of their development (e.g. pelagic vs. benthic habitat) are often neglected, especially in cases where their consideration would make experiments more laborious (Chapman 2002). Also ephippia of the \textit{D. longispina} species complex represent an experimental challenge: e.g. large amounts of the small ephippia are needed to obtain sufficient material and deal with variation in egg content and hatchability and their hydrophobic surface characteristics make it difficult to work with them in the lab.

The above mentioned points are a relevant issue as they introduce a bias into aquatic toxicological research and may lead to unrealistic predictions and risk assessments. In the present work, we overcome these shortcomings by exposing resting eggs of a natural population of the \textit{D. longispina} complex that build-up the ecologically and evolutionary important egg bank in lake sediments to a complex mix of micropollutants previously detected in the sediments of the same lake. Making use of an automated counting software and an elaborate experimental setup, we demonstrate that even with large numbers of ephippia required for these kind of experiment (a total of 254,950 ephippia), the study was conveniently handled and evidence for a significant impact of micropollutants on hatching success and mortality of hatchlings were found in replicated experiments.
Hatching success

We found a highly significant treatment effect on hatching success (p < 0.001, Table 2, Figure 1 and 2).

Table 2. Estimates and test statistics for a quasi-binomial generalized linear model examining hatching success using fixed factors treatment and experiment as explanatory variables. (null deviance: 610.27, df=119; residual deviance: 526.29, df=117, dispersion parameter: 4.438). p-values are given for individual hypothesis tests and partial F-tests.

|                | Estimate | Std. Error | t       | p>|t| | F       | p>|F| |
|----------------|----------|------------|---------|-------|---------|-------|
| Intercept      | -2.689   | 0.069      | -38.806 | < 0.001 |         |       |
| Treatment      | 0.276    | 0.076      | 3.661   | < 0.001 | 13.322  | < 0.001 |
| Experiment     | 0.172    | 0.075      | 2.285   | 0.024  | 5.166   | 0.025 |

Figure 1: Boxplots of hatching success for control and exposure treatments in the two experiments (A and B). Each boxplot is based on 30 observations at four time points. Whiskers indicate 1.5 x interquartile range of the upper and the lower quartile, respectively. Outliers are shown as open circles.

Surprisingly, hatching success increased in both experiments when ephippia were exposed to a mixture of organic contaminants. While a few studies have reported a negative impact of organic and inorganic contaminants on hatching success (Bagshaw et al. 1986; Rafiee et al. 1986; Angeler et al. 1986; Angeler et al. 1986).
2005; Marcial et al. 2005; Jiang et al. 2007; Marcial & Hagiwara 2007; Navis et al. 2013), this is the first time that an increase in hatching has been observed. Three possible explanations for this finding are discussed.

![Figure 2: Mean cumulative hatching success over time for control and exposure treatments in the two experiments (A and B). Error bars indicate standard errors of means (SEM).](image)

First, one or several compounds used in the experiment may interfere with physiological pathways that control dormancy. They may directly interact with the cellular machinery that maintains cell cycle arrest or affect the sensitivity of signaling pathways that transmit hatching cues. Unfortunately, practically nothing is known about the physiology of dormancy control and the involved regulatory pathways. However, Navis et al. (2013) found evidence that the known endocrine disruptor fenoxycarb has a detrimental effect on hatching showing that endocrine disruptors can affect hatching success. From our selected compounds, triclocarban has been classified as a new type of endocrine disruptor since it amplifies the transcriptional activity of steroid hormones and their receptors (Chen et al. 2008). Interestingly, triclocarban has indeed been shown to stimulate embryo production in the freshwater mudsnail *Potamopyrgus antipodarum* at relevant environmental concentrations (Giudice & Young 2010). Furthermore, having a similar structure to triclocarban, triclosan has been reported to act as endocrine disruptor in fish (Raut & Angus 2010) as well as an inhibitor of lipid biosynthesis, preventing bacteria from building cell membranes and interfering with
other vital functions (McMurry et al. 1998). In addition, the musk fragrance tonalide, the UV filter octocrylene and the pesticides terbutryn, prochloraz and propiconazole are also suspected to act as endocrine disruptors (Svetlana & Eva 2012).

A second possible explanation is environmentally cued hatching (Warkentin 2011). Hatching of *Daphnia* resting eggs is known to be triggered by signals indicating favorable conditions for development and reproduction in the pelagial, e.g. photoperiod and temperature (Vandekerkhove et al. 2005). There are, however, also examples in the animal kingdom where early hatching is initiated when conditions become unfavorable for the egg (“escape hatching”), e.g. in the presence of egg predators or fungal growth (Warkentin 2011 and references therein). As a result, resting eggs may, in principal, also be capable of sensing a decrease of their chance of survival in the sediment and escape by terminating diapause and hatching. Interestingly, this possibility has to our knowledge not been considered or tested for *Daphnia* resting eggs so far. Such an escape hatching response may for example have been triggered via cellular damage through the chemicals in our experiment.

Third, an increase in hatching could also be explained by a more indirect effect, i.e. reduction of microbial growth caused by the micropollutants mix. Damaged resting eggs infested with fungi or bacteria are regularly encountered in ephippia collected from lake sediments (Möst, M., personal observation), and microbial growth is therefore likely to interfere with the development of hatchlings and hatching success. Furthermore, four of the compounds used in the experiment (propiconazole, prochloraz, triclocarban and triclosan) are known to have antimicrobial activity (see Table 1). Although we used double autoclaved lake water and sterile tubes in all our assays and no obvious microbial growth during hatching was observed, microbial growth had been observed in pre-tests after several days of incubation. Therefore, inhibition of microbial growth by biocides remains as a likely explanation for the increase in hatching success.

In addition to the effect of organic contaminants, a weak significant effect for the experiment (p-value 0.025) explained by a higher hatching success in the second experiment was found (Figure 1 and 2). One possible explanation for this observation may be seasonality. The first experiment was conducted in December, while the second experiment was conducted in March, which is the start of the growing season and onset of hatching in nature (Carvalho & Wolf 1989; Cáceres 1998; Hairston et al. 2000; Brendonck & De Meester 2003).

**Hatchling mortality**

Mortality of hatchlings was increased in the experiments with the mix of chemicals, however, the size of the effect differed between experiments resulting in a significant interaction (p < 0.001, Table 3, Figure 3 and 4). In total, we found 648 dead and 850 alive hatchlings in the control versus 1712 dead and only 67 alive in the exposure treatment.
Table 3. Estimates and test statistics for a quasi-binomial generalized linear model examining mortality using fixed factors treatment, experiment and their interaction as explanatory variables. (null deviance: 1044.79, df=118; residual deviance: 294.41, df=115, dispersion parameter: 2.321). p-values are given for individual hypothesis tests and partial F-tests.

|                | Estimate | Std. Error | t     | p(|t|) | F   | p(|F|) |
|----------------|----------|------------|-------|--------|-----|--------|
| Intercept      | 1.111    | 0.138      | 8.050 | < 0.001|     |        |
| Treatment      | 0.464    | 0.192      | 2.414 | 0.017  |     |        |
| Experiment     | -1.417   | 0.174      | 8.138 | < 0.001|     |        |
| Treatment x Experiment | -2.977 | 0.327      | 9.091 | < 0.001| 89.95| < 0.001|

Figure 3: Boxplots of mortality for control and exposure treatments in the two experiments (A and B). Each boxplot is based on 30 observations at four time points. Whiskers indicate 1.5 x interquartile range of the upper and the lower quartile, respectively. Outliers are shown as open circles.

Our experiment was primarily targeted to investigate effects on hatching success but mortality was also recorded in parallel on days 7, 10, 13 and 15. Therefore, mortality here describes the proportion of dead hatchlings encountered after two and three days, respectively, and individuals may have hatched at any time during this period. As a result the design of our experiment does not allow to
disentangle an effect of the toxicants on the developing embryo in the resting egg from an effect on the hatched individuals. However, hatchlings that had died shortly after or even during the hatching process were frequently found in the exposure treatment, some showing deformations and developmental abnormalities. This observation suggests that the mixture of chemicals affected already the developing embryo in the egg. Furthermore, these results are in agreement with the reduced survival of hatchlings of *D. magna* after resting egg exposure to fenoxycarb and carbaryl (Navis *et al.* 2013).

While in both experiments mortality was higher in the exposure medium, the size of the effect differed considerably between the two experiments. This difference is due to an increased mortality in the control treatment of the first experiment, that may be attributed to subtle differences in handling or in the timing of peak hatching (i.e. hatchlings that hatched shortly after medium exchange remain in the assay tubes for up to three days without food which increases their risk of mortality) between the two experiments.

![Figure 4](image_url)

**Figure 4:** Mean mortality over time for control and exposure treatments in the two experiments (A and B). Error bars indicate standard errors of means (SEM).

The observed effect on mortality was not unexpected and can be associated with the exposure concentrations of the selected compounds. The effect concentration (EC$_{50}$) values for *D. magna* of
the studied compounds are between 20 to 200 times higher, with the exception of tonalide, triclosan and triclocarban, when compared to the measured exposure concentration (see Table 1). Tonalide has shown to have negative effects in zebrafish embryos and larvae at concentrations higher than 100 µg/L (Carlsson & Norrgren 2004). *D. magna* EC$_{50}$ values for tonalide, triclosan and triclocarban are similar or higher than the exposure concentrations used (Balk & Ford 1999; EPA 2002). The 48-h *D. magna* EC$_{50}$ values for survival and reproduction have been reported to be 390 µg/L for triclosan (Orvos *et al*. 2002; Tamura *et al*. 2012) which is close to the exposure concentration in the experiment.

**Implications**

This study reveals that organic contaminants present in large lakes have the potential to affect the function of egg banks and thereby benthic-pelagic coupling via two processes: increased hatching on one hand and increased mortality of hatchlings on the other hand. Either case may have severe consequences for the ecology and evolution of species that are dependent on the egg bank. Individuals hatched from resting eggs differ from parthenogenetically produced individuals with respect to life history traits related to population growth, physiology and their ability to cope with different food levels (Arbaciauskas & Gasiumaite 1996; Arbaciauskas & Lampert 2003). Changes in the contribution of ex-ephippial hatchlings to the pelagic population will therefore affect abundance and average life history traits of the whole population. Such an impact on the seasonal population dynamics of relevant zooplankton species is not only expected to have an effect on the ecology and survival of this species but also on the whole lake ecosystem through food web interactions (see Nasis *et al*. 2013). In addition, the egg bank also represents a storage of genetic variation produced by sexual recombination over several generations (Ellner & Hairston 1994; Brendonck & De Meester 2003; De Meester *et al*. 2006) and reduced hatching from the sediments may have severe implications on the evolutionary potential of species (Brendonck & De Meester 2003). The ecological and evolutionary consequences of decreased hatching success and hatchling fitness have also been discussed by Nasis *et al*. (2013), however, this study reveals a further and so far unobserved outcome of organic contaminants exposure of resting eggs, i.e. an increase in hatching success. *Daphnia* resting eggs do not all hatch at the same occasion (e.g. De Meester & De Jager 1993) which allows for trans-generational overlap and reducing the risk of extinction (Ellner & Hairston 1994; Gyllststrom & Hansson 2004). Increased hatching may therefore interfere with bet-hedging strategies, lead to a depletion of the egg bank and thereby increase the extinction risk of local populations (Chesson 1983; Ellner & Hairston 1994; Brendonck & De Meester 2003; Gyllstrom & Hansson 2004). Furthermore, it may affect competition between species in cases when the reaction of hatching to organic contaminants differs between species.
Which of the processes observed in our experiment, i.e. increased hatching and increased mortality, respectively, occurs and predominates in nature, depends on environmental concentrations, the mix of organic contaminants as well as exposure time, and requires further studies. We used 1,000 fold increased concentrations for the organic contaminants in our experiment to evaluate whether or not an effect in hatchability can be observed, however, future research needs to focus on environmentally more realistic scenarios. Furthermore, resting eggs can stay in the active resting egg bank for decades and therefore chronic low-dose effects are likely to be relevant. Moreover, availability and uptake of organic contaminants may be altered by the presence of sediments and the microbial flora therein. Such questions should be addressed with long-term mesocosm experiments in the future.

In conclusion, this study reveals the potential of micropollutants found in lake sediments to affect ecology and evolution of a key species in large-lake ecosystems, and highlights the urgent need for further research in this direction to assess the risk that emanates from the on-going input of organic pollutants into aquatic systems.

Acknowledgements

We thank Esther Keller for her invaluable help during the experiment, Roman Ashauer for his input in the experimental design and Christoph Tellenbach for his statistical advice. We are grateful to Sabine Navis and Luc De Meester for stimulating discussions. Funding by the Swiss National Science Foundation (SNF CR32I3_125211) is gratefully acknowledged.

References


Freitas E, Rocha O (2011) Acute toxicity tests with the tropical cladoceran *Pseudosida ramosa*: The importance of using native species as test organisms. *Archives of Environmental Contamination and Toxicology* **60**, 241-249.


**Supplementary Information**

![Chemical Concentration Graphs](image)

**Figure S1:** Stability of chemicals by light at 20 °C. Triclosan and tonalide show between 73 % and 62 % degradation, respectively, while the rest of the compounds are stable.
Chapter 4: At the edge and on the top: molecular identification and ecology of *Daphnia dentifera* and *D. longispina* in high-altitude Asian lakes

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Abstract

The occurrence of members of the highly diverse *Daphnia longispina* complex in Southern and Central Asian high-mountain lakes has been recognised for more than a century. Until now, however, no molecular data have been available for these populations inhabiting the "Roof of the World". Here, we present the first identification for *D. gr. longispina* from that region based on a molecular phylogeny. Our findings show that alpine lakes in the Pamir and Himalaya mountains host populations of widespread species of the complex, for which these are the highest known localities. A spineless morph from the Himalaya region, previously labeled as *D. longispina* var. *aspina*, was clustering tightly with *D. dentifera*, while a population from the Pamir mountain range was grouped with *D. longispina*. In addition, we analyzed ecological data available for lakes in the Khumbu region (Himalaya) to investigate ecological preferences of non-pigmented *D. gr. longispina*. The identified factors can at least partly be related to avoidance of high UV conditions by this species. We conclude that the widespread species *D. dentifera* and *D. longispina* also colonised the Asian high-mountain lakes and identify the need of further research to trace the possible effect of rapid environmental changes in this region on the diversity and ecology of high-altitude *Daphnia* populations.

Keywords: *Daphnia longispina* complex, Alpine lakes, Molecular systematics, UV radiation, 12S
Introduction

Members of the *Daphnia longispina* complex are distributed throughout the Holarctic and comprise some of the most common and ecologically important water flea species (Petrusek et al. 2008). The different taxa within this complex are difficult to distinguish based on morphological characters and frequent hybridisation and introgression between several taxa further complicates a reliable morphological identification (Petrusek et al. 2008; Dlouha et al. 2010). Within the last decades, several molecular methods have become available that allow to distinguish lineages and hybrids within the *D. longispina* complex (e.g. Wolf & Mort 1986; Billiones et al. 2004; Brede et al. 2006; Skage et al. 2007). Most recent studies have mainly focused on ecology or diversity of a few widespread species and their hybrids inhabiting large lakes, such as *D. longispina* O. F. Müller, 1776, *D. galeata* G. O. Sars, 1863, and *D. cucullata* G. O. Sars, 1862 in Europe, and *D. galeata* and *D. dentifera* Forbes, 1891 in the Eastern Palearctic and North America.

However, other species with more restricted distributions occur in the Palearctic, for example *D. lacustris*, mostly confined to Fennoscandia, but with some isolated occurrences in mountain lakes (Nilssen et al. 2007). Further, molecular methods have allowed distinguishing additional cryptic lineages in both Asia (Ishida et al. 2011; Zuykova et al. 2013) and Europe (Petrusek et al. 2008; Petrushkev et al. 2012). At least one of these lineages has very wide distribution, as it has been found in the northeast of European Russia, as well as in the Irkutsk Reservoir in Siberia, more than 3000 km apart (Petrusek et al. 2012). Another distinct clade of the *D. longispina* complex, closely related to both *D. dentifera* and *D. longispina*, has been reported by Ishida and Taylor (2007a) from Tomsk and Baikal regions of Siberia.

These findings accentuate the fact that the knowledge on the diversity of the *D. longispina* complex in Southern and Central Asia remains scarce, as most of the available data are based on morphological identification only. Some older works (e.g. Sars 1903; Uéno 1937; Brehm & Woltereck 1939) reported specific *Daphnia* morphs from this region, but linking these observations to presently recognised lineages is challenging. Nevertheless, without establishing such links, ecology and distribution of lineages only discovered by genetic analyses will also remain virtually unknown, and the wealth of data in the historical literature will be of limited use.

Despite the fact that mountain lakes are often important habitats for *Daphnia* (Manca et al. 1998; Winder & Spaak 2001; Petrushkev et al. 2007) and *Daphnia* may be important components of mountain lake foodwebs (e.g. Winder et al. 2003), these lakes are relatively understudied with respect to the diversity of *Daphnia* lineages they may contain. Various studies, however, provide evidence that many mountainous regions harbor a substantial number of endemic cladoceran taxa (e.g. Kotov et al. 2010; Van Damme & Eggermont 2011) and that *Daphnia* populations interesting
from biogeographic (e.g. Petrusek et al. 2007) as well as evolutionary (e.g. Mergeay et al. 2008; Dufresne et al. 2011) perspectives can be discovered in such environments. On the other hand, finding of special daphnid lineages in alpine lakes is not a general rule. For example, an analysis of the *D. longispina* complex from lakes in eight mountain ranges of southeastern Europe revealed the presence of a single *Daphnia* species, though its populations were substantially differentiated (Hamrová et al. 2012).

Having obtained samples of *Daphnia* useful for genetic analyses from remote lakes of the mountain ranges of South and Central Asia (Himalaya and Pamir; Fig. 1), we therefore asked the question whether we can find cryptic lineages or rather the widely distributed members of the *D. longispina* complex in such habitats.

*D. longispina* and similar forms have been reported from Central Asian mountains by numerous authors, for example, from lakes in Tibet and the Altai and Pamir mountains by Sars (1903), from Tibet by Daday (1908), from Pamir by Vereschagin (1923) or Rylov (1930), and from the Chinese Khingan mountain range by Uéno (1937). Various forms from these regions have also been formally described, usually as specific varieties but occasionally also as distinct species, e.g. *D. sonkulensis* Manuilova, 1964 from Tian-Shan mountains. Sars (1903) described four new varieties for *D. longispina* for Central Asia, among them *D. longispina* var. *turbinata* from the Altai (later considered by some authors as a distinct species; e.g. Glagolev (1995); Manuilova (1964)). In the description itself, Sars (1903) noted that var. *turbinata* was very close to certain forms of *D. lacustris* (considered by himself at that time as a subspecies of *D. longispina*). He also reported from Tibet *D. longispina* f. *caudata*, a form that was shown to be mostly conspecific with *D. lacustris* in Fennoscandia (Nilssen et al. 2007). Several expeditions to North India and Tibet found a "pale" *D. gr. longispina*, often referred to as *D. longispina* or *D. longispina* var. *aspina* Vereschagin, 1911, and pigmented *Ctenodaphnia*, variously described as *D. tibetana* Sars, 1903, *D. fusca* Gurney, 1906, *D. pamirensis* Rylov, 1930 or *D. himalaya* Manca, Martin, Penalva-Arana & Benzie, 2006 (e.g. Hutchinson 1937; Brehm & Woltereck 1939; Löffler 1969; Dumont & Velde 1977; Manca et al. 1994).

A revision of the systematics of the pigmented *Ctenodaphnia* from this region is in progress (V. Kořínek, pers comm.). Therefore, we focus here on the molecular systematics of the non-pigmented *D. gr. longispina*, presenting phylogenetic analyses for individuals collected from the Himalaya (Nepal) and the Pamir (Tajikistan) mountain ranges. We hypothesized that the distinctively different morphotypes collected in these two regions may represent different species.

In addition, we aimed to better understand the ecological factors driving the distribution patterns of non-pigmented *D. gr. longispina* in the harsh environment of the Himalaya range. Therefore, we collected and analyzed published data for lakes in the Khumbu region in northeastern
Nepal (Manca et al. 1994, 1998; Tartari et al. 1998; Lacoul & Freedman 2005; Sommaruga & Casamayor 2009; Sommaruga 2010; Sharma et al. 2012). These lakes are situated between 4700 m and 5500 m a.s.l., are fishless, and are often inhabited by either non-pigmented *D. gr. longispina* and/or melanized *Ctenodaphnia*. The daphnids frequently co-occur with the calanoid copepod *Arctodiaptomus jurisowitchi* Löffler, 1968, and various chydorid species (for further information see Löffler 1969; Manca et al. 1998; Tartari et al. 1998). Löffler (1969) already observed that the "pale" species seems to prefer turbid or deep lakes whereas the pigmented one predominates in shallow and clear lakes, a pattern ascribed to differences in UV resistance. Furthermore, Sommaruga (2010) demonstrated the relevance of UV protection mechanisms for *A. jurisowitchi* in some of these lakes. However, there are many other factors that may potentially shape the distribution of different *Daphnia* species in high-elevation lakes besides UV stress, such as low temperatures, low nutrient/food concentrations, high suspended particulate matter content in lakes with glacier influence, and interspecific competition.

In the current study, we aimed to increase the knowledge on biogeography and ecology of the *D. longispina* complex by reconstructing the molecular phylogeny of two different morphotypes from Pamir and Himalayan high mountain lakes. Furthermore, we examined ecological preferences of *D. gr. longispina* in lakes of the Himalayan Khumbu region and discuss our findings also with respect to the on-going environmental change in this habitat.

**Material and Methods**

**Sampling sites and populations**

Samples from the Himalaya region were collected from Himalayan Lake Piramide Inferiore (LCN10, 27°57.8’N, 86°48.7’E, 5067 m a.s.l.), Nepal (Khumbu region) on October 14, 2010. A detailed description of the sampling area is given in Tartari et al. (1998). Morphologically, individuals from this population fit the characteristics of *D. longispina* var. *aspina*, as reported from Himalayan lakes (Fig. 2a,b). Phenotypically similar *Daphnia* is common in the region, inhabiting numerous alpine lakes (Löffler 1969; Manca et al. 1998).

The population from Tajikistan was sampled on August 18, 2011 in the Pamir mountains from Lake Rangkul (38°28’N, 74°14’E, 3789 m a.s.l.), Tajikistan. Phenotypically, it seems close to *D. longispina* varieties *caudata* or *turbinata* as reported by Sars (1903) from Central Asia (see Fig. 2c). No other similar *Daphnia* was found in samples collected from smaller water bodies of the region (M. Slusarczyk & V. Kořínek, unpubl. data). The samples were preserved in ethanol and thus suitable for DNA analyses.
Microphotography

The overall phenotype of the animals from the two regions was documented by microphotography. Specimens preserved in ethanol were stained after Kořínek (1999) with lignin pink and chlorazol black E dyes for 24 h, and subsequently either directly photographed or dehydrated with 2,2-dimethoxypropane for 10-15 min, then transferred into xylene, and mounted in Canada balsam. Sixty-four shots were taken at various focus plains by a Nikon D3100 camera mounted on an Olympus BX51 microscope with differential interference or phase contrast, and merged together using the software Helicon Focus 5.2.7. To document the Himalayan populations, better preserved
individuals from Lake Gokyo (LCN75; 27°57’N, 86°41.5'E) were selected, which are both phenotypically and ecologically very close to those from Lake Piramide Inferiore (12 km apart).

**Molecular analyses**

DNA extraction from ethanol-preserved individuals and amplification of fragments of the 12S rRNA (12S) and NADH2 (ND2) genes, as well as of the internal transcribed spacer region 1 (ITS-1) were done according to previously published protocols. DNA was extracted from five parthenogenetic females from the Himalayan sample following the HOTShot protocol (Montero-Pau et al. 2008) and from two individuals from Tajikistan using the proteinase K protocol as described by Schwenk et al. (1998).

A ca. 600 bp fragment of the mitochondrial 12S rDNA was amplified using primers and protocols from Taylor et al. (1996). Based on findings from Eastern Palearctic by Ishida et al. (2011), we also tested for possible traces of past hybridisation events and introgression in the Himalayan population. For this purpose, we additionally amplified a ca. 1,000 bp fragment of the mitochondrial ND2 gene and a ca. 760 bp one of the nuclear ITS-1 region, using also the primers and protocols previously used in several studies for ND2 (Ishida et al. 2006; Ishida & Taylor 2007a,b), as well as for ITS-1 amplification (Taylor et al. 2005).

The PCR products were purified and Sanger-sequenced in both directions by commercial sequencing services (Microsynth AG, Switzerland, and Macrogen Inc., Korea). The chromatograms were checked visually for possible scoring errors, and the resulting sequences submitted to GenBank (accession numbers JX446618-JX446621). We obtained sequences of 562 bp fragments of the mitochondrial 12S gene for all analyzed individuals, and additionally a 937 bp long fragment of the mitochondrial ND2 gene as well as a 762 bp fragment of the nuclear ITS-1 marker for the five individuals from the Himalayan locality.

The sequences of both mitochondrial genes were first compared with those representing known lineages of the whole *D. longispina* complex. For the final 12S alignment, selected sequences for *D. longispina* and *D. dentifera*, representing a wide range with respect to geographic distribution and genetic diversity, were used (Table S1). The ND2 alignment was built from a subset of sequences used in Ishida and Taylor (2007a), kindly provided by Seiji Ishida, using *D. cucullata* and *D. galeata* (GenBank accession numbers: DQ980402 and DQ980251) as an outgroup. The ITS-1 sequences were aligned with the haplotypes published by Ishida et al. (2011). Alignment building, nucleotide substitution model selection, and phylogenetic tree reconstruction were done with MEGA 5 (Tamura et al. 2011).

Nucleotide substitution models were selected applying the Bayesian Information Criterion. The Tamura-Nei model of DNA evolution (Tamura & Nei 1993) with gamma distributed rate
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Heterogeneity was chosen for reconstructing of 12S and ND2 phylogenetic trees using the maximum likelihood (ML) method. Similarly, a ML tree was built for the ITS-1 sequences using the Kimura 2-parameter model (Kimura 1980) with gamma distributed rate heterogeneity. In addition, a neighbor-joining tree with evolutionary distances computed using the Maximum Composite Likelihood method (Tamura et al. 2004) was constructed for each gene. The node support in each tree was assessed by 1,000 bootstrap replicates.

**Statistical analyses of ecological data**

A binary logistic regression analysis to identify factors explaining the presence of the non-pigmented *D. gr. longispina* in lakes in the Himalayan Khumbu region was conducted using the statistical software R (R Development Core Team 2012). Data from 20 lakes in this region were collected from several publications (Manca et al. 1994, 1998; Tartari et al. 1998; Lacoul & Freedman 2005; Sommaruga & Casamayor 2009; Sommaruga 2010; Sharma et al. 2012). Variables collected were presence/absence of the *D. gr. longispina* and the pigmented *Ctenodaphnia*, turbidity (milky/clear), water depth, lake area, altitude, electrical conductivity, and total concentrations of phosphorus (TP), silicate, and calcium. Logistic regression was computed using the function *glm*, stepwise model selection based on Akaike Information Criterion (AIC) was run with the function *step*, and likelihood ratio tests (LRT) for the significance of single terms in the selected models were done with the function *drop1*. Values for all quantitative variables were log-transformed to correct for positive skewness of their distributions.

**Results**

**Identification of alpine D. gr. longispina**

Based on the grouping of newly obtained sequences with those of known *Daphnia* species, we identified the individuals from the Himalayan locality (Fig. 2a,b) as *D. dentifera* and those from Tajikistan (Fig. 2c) as *D. longispina* sensu stricto. All individuals from the same location were identical in the analyzed genes.

The overall topologies of the phylogenetic trees based on the mitochondrial 12S (Fig. 3) and ND2 (Figure S1) genes are congruently supporting the existence of three closely related but distinct clades in Central Asia: *D. dentifera*, *D. longispina* and a third clade from Siberia (Ishida & Taylor 2007a). The phylogenetic reconstructions reflected different levels of variation present within these clades, the *D. dentifera* being most variable, with numerous distinct intraspecific lineages.

The nuclear ITS-1 phylogeny also identified the Himalayan sample as *D. dentifera*, and its congruent position in the nuclear and mitochondrial phylogenies revealed no indications for introgression or incomplete lineage sorting (Figure S2).
Ecological preferences of Himalayan D. gr. longispina

The results of our statistical analysis suggest that the non-pigmented D. gr. longispina in the Khumbu region preferentially inhabit deep lakes at the lower range of total phosphorus concentrations and conductivity, and with glacier influence to some extent (i.e., presence of minerogenic turbidity).

Model selection resulted in a best binary logistic regression model with depth, total phosphorus, turbidity, and conductivity as explanatory variables for the presence of the non-pigmented D. gr. longispina (LRT: $\chi^2 = 16.183$, df = 4, $p < 0.003$; Nagelkerke $R^2 = 0.629$; AIC: 21.34). According to the model, the likelihood for a lake being inhabited by the non-pigmented D. gr. longispina increases with greater depth and decreases with higher total phosphorus concentrations and conductivity. Lakes with some glacier influence were more likely to contain non-pigmented D. gr. longispina. Though minerogenic turbidity and especially conductivity were weakly significant, model comparison based on likelihood ratio tests suggested to keep these parameters in the model. Model parameters and likelihood ratio test statistics are summarized in Table 1.

In the second best model, only depth and total phosphorus were kept as significant explanatory variables, with depth as the predictor of higher significance (LRT: $\chi^2 = 9.335$, df = 2, $p < 0.01$; Nagelkerke $R^2 = 0.377$; AIC: 24.19; see Table 2).
Figure 3: Maximum likelihood phylogenetic tree for 12S rRNA. The new haplotypes from the Himalaya and Tajikistan are highlighted in bold. Numbers on major branches are bootstrap values (1,000 replications) obtained for maximum likelihood (ML) and neighbor-joining (NJ) phylogenetic analyses (ML/NJ). The scale bar indicates the number of substitutions per site.
Table 1. Estimates and likelihood ratio test statistics for a binary logistic regression model examining absence/presence of the non-pigmented Daphnia in alpine lakes of the Khumbu region using water depth (log(Depth)), total phosphorus concentrations (log(TP)), presence of minerogenic turbidity (clear/milky), and electrical conductivity (log(Cond)) as explanatory variables. (null deviance: 27.526, df=19; residual deviance: 11.343, df=15)

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<th>LRT</th>
<th>p (&gt;χ²)</th>
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Table 2. Estimates and likelihood ratio test statistics for a binary logistic regression model examining absence/presence of non-pigmented Daphnia in alpine lakes of the Khumbu region using water depth (log(Depth)) and total phosphorus concentrations (log(TP)) as explanatory variables. (null deviance: 27.526, df=19; residual deviance: 18.190, df=17)

<table>
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Discussion

Identification of alpine D. gr. longispina

The phylogenetic analyses revealed that D. gr. longispina from Lake Piramide Inferiore in the Nepalese Himalaya, formerly referred to as Daphnia longispina var. aspina, actually constitutes a lineage within the D. dentifera clade. Furthermore, the 12S phylogeny clearly identified the individuals from Lake Rangkul (Tajikistan) as D. longispina. We thus confirmed that the two morphotypes represented different species but we revealed neither endemic nor rare species. On the contrary, both species are among the most widespread ones in the D. longispina complex, being confirmed genetically in at least two biogeographic regions each (Palearctic and Holarctic in case of D. dentifera; Ishida & Taylor 2007a; and Palearctic and Ethiopian in case of D. longispina; Petrush et al. 2008). Our results further extend our knowledge on the distribution of both species. To our knowledge, the Himalayan Lake Piramide Inferiore represents not only the southern- and westernmost but also the highest locality reported for D. dentifera. Similarly, Lake Rangkul is the
south-easternmost and also the highest known locality for *D. longispina*, evidenced by molecular data.

In detail, all three phylogenies, 12S, ND2, and ITS-1, grouped the Himalaya sample together with *D. dentifera* sequences from Japan and North America. We did not find evidence for past hybridisation or introgression in our data (unlike in Japanese *D. dentifera* populations; see Ishida *et al.* 2011). It is likely that most or all populations with the *D. longispina* var. *aspina* morphotype that have been reported from several other lakes in the Khumbu region (Manca *et al.* 1994, 1998; Tartari *et al.* 1998) actually represent *D. dentifera*, and the mountain populations in the Himalayas do not coexist with other species of the same complex. However, given the high phenotypic plasticity and frequency of hybridisation events in the *D. longispina* complex and the resulting difficulties for morphological identification, this prediction should be further tested by more detailed studies.

Although some of the morphs similar to animals from Lake Rangkul in Pamir, reported by Sars (1903) from Central Asian mountains, were considered by this author close to *D. lacustris* (presently proven to be a distinct species), we unambiguously identified the Tadjik population as belonging to *D. longispina*. This species is particularly widespread in European mountain ranges (Petrusek *et al.* 2007; Petrushik *et al.* 2008; Hamrová *et al.* 2012), and occurs in mountain lakes in numerous morphs that had been distinguished under separate names (as varieties and forms) in the early 20th century (see e.g. Petrushik *et al.* 2007). It is thus possible that various varieties reported from Central Asia in historical literature are also conspecific with *D. longispina*.

However, it is undeniable that wider diversity in the *D. longispina* complex can be found in this region, including lineages with unclear taxonomic status. A mitochondrial lineage closely related to both *D. longispina* and *D. dentifera* (based on ND2 analysis) has been reported from six widely distributed Siberian localities by Ishida and Taylor (2007a). Presumably, the same clade is apparent also in our 12S tree, represented by sequences from several localities in the Chany Lake basin in southwestern Siberia (Zuykova *et al.* 2013). Several other haplotypes from the Chany lake basin, however, can be found within the narrow *D. longispina* clade, indicating the coexistence of these two lineages in Siberia. Furthermore, other common species of the *D. longispina* complex, such as *D. galeata* and *D. cucullata*, are also distributed throughout Siberia (Ishida & Taylor 2007b; Zuykova 2010) and highly divergent members of the complex that cannot be at present linked to existing names, are found there as well (Petrusek *et al.* 2012; Zuykova *et al.* 2013). It is thus possible that some of these species also at least occasionally colonise high-altitude mountain lakes.

**Ecological preferences of Himalayan D. gr. longispina**

Another aspect of this work was the analysis of ecological data available for lakes in the Khumbu region to identify factors influencing the occurrence of the *D. gr. longispina* (identified as *D. dentifera*
for Lake Piramide Inferiore in the present study). Logistic regression models on presence/absence data for 20 lakes identified water depth, total phosphorus concentration (TP), minerogenic turbidity and conductivity as significant predictors, with depth and TP being the most significant ones.

The finding that greater water depth increases the likelihood that the non-pigmented *D. gr. longispina* inhabits a lake is not surprising. In contrast to the melanized *Ctenodaphnia*, *D. gr. longispina* from this region do not contain photoprotective compounds (Sommaruga 2010) in their body wall, except for pigmentation of the ephippia, and the importance of a deep water column as a refuge from ultraviolet radiation has been discussed in previous works already (Löffler 1969; Manca et al. 1994, 1998; Sommaruga 2001, 2010). In addition, deeper lakes are also better buffered against severe disturbances (e.g. freezing to the bottom, drying up) and might therefore constitute a more stable habitat in this harsh environment.

Interestingly, low TP was also a strong predictor for the presence of the *D. gr. longispina*. It is known for some members of the *D. longispina* complex that they can populate ultra-oligotrophic lakes, where they may even have competitive advantages compared to other *Daphnia* species (Rellstab & Spaak 2007; Spaak et al. 2012). However, the outcome of the statistical model did not change when including the most likely competitor in this system, the melanized *Ctenodaphnia*, as an additional variable. Actually, both pigmented and unpigmented *Daphnia* at least occasionally coexist in high-altitude Asian lakes (including e.g. Lake Rangkul in Pamir from which we analyzed *D. longispina* population), so it is clear that their encounter does not necessarily lead to competitive exclusion. It should be also mentioned that some of the measured TP values are close to the detection limit of the analytical method used. Consequently, the relevance of TP concentrations for *Daphnia* distribution in these lakes remains an open question for further research.

Löffler (1969) already emphasized the potential importance of turbidity for the occurrence of the non-pigmented *D. gr. longispina* in shallow ponds and attributed that observation to its protecting role from UV radiation. Nevertheless, it seems counterintuitive that substantial minerogenic turbidity from glaciers could be beneficial for *Daphnia*, since several studies have shown the detrimental impact of high suspended particle concentrations on *Daphnia* (e.g. Koenings et al. 1990; Kirk 1991; Rellstab & Spaak 2007). The suspended particle concentrations measured in three lakes (Giardino et al. 2010) classified as turbid by Tartari et al. (1998) furthermore suggest that also lakes with relatively low minerogenic turbidity had been categorized as turbid or that turbidity has decreased in those lakes. Interestingly, the *D. gr. longispina* has also been reported from one shallow and transparent lake (LCN40, see Tartari et al. 1998), which was explained by the benthic growth of freshwater mosses or filamentous algae serving as a refuge for zooplankton (Manca et al. 1994, 1998; Manca & Comoli 2004). Similar observations have been made for the copepod *Arctodiaptomus*
**jurisowitchi**, inhabiting a shallow high-altitude transparent pond colonised by filamentous algae (Sommaruga 2010).

In summary, the factors identified by the model as important for *Daphnia* distribution can at least partly be attributed to UV stress and habitat stability. However, experimental research would be needed to elucidate their role.

**Conclusion**

The individuals from the *D. longispina* complex collected from Southern and Central Asia were identified as *D. longispina* and *D. dentifera* based on molecular phylogenies. That suggests that the distributional range of these species is considerably wider than assumed so far. Several factors explaining the presence of non-pigmented *D. gr. longispina* in high-altitude mountain lakes of the Khumbu region were identified. The significant effects of water depth and turbidity are well in accordance with previous studies, relating the presence of *D. gr. longispina* to conditions protecting from UV radiation. A shift in ecological conditions due to the rapid retreat of glaciers in this region (Cruz et al. 2007) may potentially have negative impacts on *D. gr. longispina* populations. On the one hand, the increasing glacial run-off may increase minerogenic turbidity to levels that preclude the existence of *Daphnia* (Koenings et al. 1990). On the other hand, as soon as lakes lose their connection to the glacial drainage systems, their transparency and consequently UV stress for non-pigmented *Daphnia* may increase.

Altogether, the study has revealed that already a limited number of samples from remote mountain areas can substantially improve our understanding of the phylogeny and distribution of *Daphnia* lineages. The fact that we found the widespread *D. dentifera* and *D. longispina* does not preclude the potential presence of cryptic lineages in this area. In the light of recent findings on the high cryptic lineage diversity in this complex (Petrusek et al. 2008; Ishida et al. 2011; Petrusek et al. 2012; Zuykova et al. 2013) investigating these mountain ranges seems thus to be even more interesting. Specifically, their remoteness and extreme habitats render the presence of locally adapted, so far undiscovered lineages of *Daphnia* as well as other cladocerans in these regions likely.

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K²-CNR) in collaboration with the Nepal Academy of Science and Technology, and thanks to contributions from the Italian National Research Council and the Italian Ministry of Foreign Affairs. Phylogenetic analyses were performed with the help of the Genetic Diversity Centre of ETH Zurich.

**References**


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Vereschagin GY (1923) Zamekta o presnovodnoi faune Pamira. Notiz über die Süßwasserfauna des Pamirs. Izvestii Rossijskogo gidrobiologicheskogo instituta 6, 21-40 [in Russian with German summary].


## Supplementary Information

**Table S1.** Information on sequences used for the 12S phylogeny (ID, country, location, GenBank accession numbers).

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Figure S1: Maximum likelihood phylogenetic tree for ND2. The new haplotype from the Himalaya is highlighted in bold. Numbers on major branches are bootstrap values (1,000 replications) obtained for maximum likelihood (ML) and neighbor-joining phylogenetic analyses (ML/NJ). The scale bar indicates the number of substitutions per site.
Figure S2: Maximum likelihood phylogenetic tree for *ITS-1*. The new haplotype from the Himalaya is highlighted in bold. Numbers on major branches are bootstrap values (1,000 replications) obtained for maximum likelihood (ML) and neighbor-joining phylogenetic analyses (ML/NJ). The scale bar indicates the number of substitutions per site.
Chapter 5: Human-caused eutrophication affects taxonomic composition and patterns of hybridisation in the *Daphnia longispina-galeata-cucullata* complex.

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Abstract

Eutrophication constitutes a primary problem for lentic water bodies world-wide and has a strong impact on lake ecosystems, e.g. increased productivity, shifts in species composition and food web effects. The hybridising *Daphnia longispina-galeata-cucullata* species complex comprises taxa, occupying a critical role in food webs in many peri-alpine lakes north and south of the European Western Alps. Paleogenetic studies in several lakes north of the Alps identified *D. longispina* as the native species and demonstrated the invasion of *D. galeata* followed by taxonomic shifts and pervasive hybridisation and introgression during a period of trophic changes. A recent field survey revealed a dominance of *D. galeata* and *D. longispina x galeata* hybrids in lakes south of the Alps leading to the hypothesis that *D. galeata* is native to the South. Our paleogenetic study of two Italian lakes reconstructs trophic changes and associated taxonomic shifts and hybridisation patterns south of the Alps over several decades. We observe drastic changes in taxonomic composition and hybridisation of all three parental species over time and present evidence that *D. longispina* is in fact also native to southern peri-alpine lakes. We discuss the observed shifts in taxonomic composition in the context of known ecological characteristics of the species and the drastic environmental changes in both lakes over several decades. We conclude that changes in food quantity and quality and size-selective fish predation pressure represent the most likely explanation for the temporal succession of the three different parental species and their hybrids. Moreover, we propose that the role of *D. cucullata* for the evolutionary trajectories of this species complex has been underestimated so far.

**Keywords:** *Daphnia longispina* species complex, hybridisation, eutrophication, size-selective fish predation, paleogenetics, sediments
Introduction

Cultural eutrophication, i.e. enrichment of aquatic systems with nutrients caused by human population growth, increasing industrialisation and agriculture, has become the primary problem for surface waters worldwide (e.g. Schindler 2006; Schindler 2012). The increased input of phosphorus and nitrogen into lakes has drastic effects on lentic ecosystems: increased productivity, algal and cyanobacterial blooms, shifts in species composition and biodiversity, major food web disturbances (e.g. occasional fish kills) and drinking water quality issues (see Correll 1998; Schindler 2006; Smith & Schindler 2009; Schindler 2012). Eutrophication can also pave the way for biological invasions (Weide et al. 1997; Jankowski & Straile 2003; Chase & Knight 2006; Brede et al. 2009; Rellstab et al. 2011; Kokociński & Soininen 2012; Spaak et al. 2012) and affect the evolutionary fate of species, e.g. by promoting hybridisation (Seehausen et al. 1997; Seehausen et al. 2008; Brede et al. 2009; Vonlanthen et al. 2012). For many lakes, especially in developing countries, eutrophication constitutes a highly topical issue (Nyenje et al. 2010; Vörösmarty et al. 2010; Pernet-Coudrier et al. 2012; Schindler 2012). In most European and several other developed countries, governmental regulations, the installation of wastewater treatment plants and successful remediation measures have resulted in a reduction of anthropogenic nutrient input (i.e. re-oligotrophication) and the restoration of the natural trophic state of several lakes (Schindler 2012).

Such changes in trophic conditions can affect all trophic levels of an aquatic food web. *Daphnia* spp., being planktonic grazers and an important food source for fish and invertebrate predators, occupy a key position in lentic food webs and therefore represent an ideal model to study the effects of human-made trophic changes (Lampert 2011). Most *Daphnia* reproduce by cyclic parthenogenesis, i.e. they reproduce asexually during most of the growing season and switch to sexual reproduction in particular when conditions deteriorate, e.g. because of reduced food availability or lowered temperature. The sexually produced resting eggs are encased by protective sheets that form a so-called ephippium. Ephippia may either float on the water surface and be dispersed by water fowl, insects and wind or sink down to the lake bottom where they contribute to the build-up of a resting egg bank (Zaffagnini 1987; Ebert 2005; Lampert 2011). The resting egg bank plays an important role for dispersal as well as ecology and evolution of species. A portion of ephippia sink to deep parts of a lake where they do not receive hatching stimuli (e.g. light and increased temperatures), are covered with sediments and represent a biological archive (for reviews see Hairston & Kears 2002; Brendonck & De Meester 2003; Gyllstrom & Hansson 2004). Although resting egg banks may not always exactly reflect the extant pelagic population, e.g. because of differential sexual investment of different taxa and clones (Jankowski & Straile 2003; Keller & Spaak 2004), they have been shown to provide a useful record of taxonomic and evolutionary changes over
the long-term, e.g. successful invasions of species, hybridisation (Weider et al. 1997; Jankowski & Straile 2003; Brede et al. 2009; Rellstab et al. 2011) or adaptation (Hairston et al. 1999; Cousyn et al. 2001; Orsini et al. 2013).

Currently, the most common *Daphnia* spp. in many European lakes belong to the *D. longispina* species complex (e.g. Schwenk & Spaak 1995; Petrusek et al. 2007; Keller et al. 2008; Petrusek et al. 2008a; Thielsch et al. 2009; Yin et al. 2010; Hamrová et al. 2012). This complex comprises at least six species and several cryptic lineages in Europe (Petrusek et al. 2008a; Petrusek et al. 2012) and hybridisation is frequently observed where species occur in syntopy (Schwenk & Spaak 1995; Keller et al. 2008; Brede et al. 2009). The dominating taxa in the peri-alpine lakes that are subject of this study are *D. longispina* O. F. Müller, 1776 (formerly known as "D. hyalina", see Petrusek et al. 2008a), *D. galeata*, G. O. Sars, 1863, *D. cucullata* G. O. Sars, 1862, and their interspecific hybrids (Schwenk & Spaak 1995; Keller et al. 2008). Morphological identification is complicated by high phenotypic plasticity and hybridisation. Therefore, several genetic marker systems have been developed to distinguish parental taxa and their hybrids (e.g. Wolf & Mort 1986; Gießler 1997; Schwenk et al. 2000; Billiones et al. 2004; Brede et al. 2006; Skage et al. 2007).

Despite evidence for hybridisation, introgression and reticulate evolution (e.g. Wolf & Mort 1986; Schwenk & Spaak 1995; Spaak 1996; Gießler 1997; Jankowski & Straile 2004; Brede et al. 2009; Gießler & Englbrecht 2009), all three taxa represent well-recognised, genetically distinct species with considerable levels of mitochondrial and nuclear differentiation (Schwenk et al. 2000; Petrusek et al. 2008a). No evidence for mate choice has been found so far, however, a certain degree of reproductive isolation exists. First, there is temporal separation of sexual reproduction between parental species, with *D. galeata* predominately reproducing sexually in spring and *D. longispina* and *D. cucullata* in autumn (Spaak 1995a; Jankowski & Straile 2004). Second, reduced mating and hatching success has been shown for crosses between *D. galeata* and *D. cucullata* (Schwenk et al. 2001) and hybrids between *D. galeata* and *D. longispina* exhibit reduced sexual fitness (Keller & Spaak 2004; Keller et al. 2007).

Furthermore, there is ecological differentiation between taxa in this species complex with respect to food quantity (Gliwicz 1990; Gliwicz & Lampert 1990; Weider & Wolf 1991; Weider 1993; Boersma & Vijverberg 1994a,b; Spaak et al. 2012) and food quality requirements (Gliwicz & Lampert 1990; Seidendorf et al. 2007), vulnerability to vertebrate and invertebrate predation (Spaak 1995a; Spaak & Hoekstra 1995, 1997; Spaak et al. 2000; Spaak & Boersma 2001; Declerck & Meester 2003; Spaak & Boersma 2006; Wolinska et al. 2007), spatial distribution (Stich & Lampert 1981; Weider & Stich 1992; Flößner 2000; Seda et al. 2007) and susceptibility to parasites (Wolinska et al. 2006, 2007). According to Flößner (2000), *D. longispina* ("hyalina") prefers oligotrophic lakes, *D. galeata*
prefers meso- to eutrophic conditions and *D. cucullata* occurs in eutrophic systems. This is supported by several field studies (e.g. Keller et al. 2008; Petrush et al. 2008b; Brede et al. 2009; Rellstab et al. 2011) and experimental studies that have shown that *D. longispina* can cope with low food levels typical for oligotrophic conditions while *D. galeata* and *D. cucullata* require higher food levels to sustain populations (Gliwicz 1990; Weider 1993; Spaak et al. 2012). In addition, *D. cucullata* has the potential to efficiently use bacteria as food source (Geller & Muller 1981) and shows increased competitive abilities in the presence of filamentous cyanobacteria (Gliwicz & Lampert 1990). Well-documented differences between taxa with respect to their vulnerability to fish predation are also known, with *D. cucullata*, the smallest of the three species, being the least vulnerable to size-selective fish predation (Spaak & Hoekstra 1997; Spaak et al. 2000; Spaak & Boersma 2006). Moreover, some indications from life history parameters and field observations exist, suggesting that *D. galeata* might be better adapted to fish predation than *D. longispina* (for details see Spaak et al. 2012). Strong support for the role of fish predation and food levels for ecological separation of the different taxa of the *D. longispina-galeata-cucullata* species complex comes from a field study of canyon-shaped reservoirs. These reservoirs exhibit a longitudinal gradient of high fish predation and high food conditions to low fish predation and low food conditions and *Daphnia* species and hybrids distribution along this gradient is in agreement with their ecological characteristics described above, a pattern comparable to terrestrial hybrid zones (Seda et al. 2007; Petrush et al. 2008b).

Hybrids are mainly produced locally (Spaak 1997; Petrush et al. 2008b; Yin et al. 2010) and often show intermediate characteristics (e.g. Weider & Wolf 1991; Weider 1993; Schwenk & Spaak 1995; Spaak & Hoekstra 1995). They have also been found to combine traits of both parental species that are advantageous under specific environmental conditions (e.g. high intrinsic rate of increase and smaller body size under intermediate fish predation pressure) and can lead to the dominance of hybrids during certain periods (“temporary hybrid superiority hypothesis”, Spaak & Hoekstra 1995, 1997).

Paleogenetic reconstructions of *Daphnia* resting egg banks in five peri-alpine lakes (Lake Constance, Greifensee, Thun, Brienz and Walensee) situated north of the European Western Alps revealed that during a phase of eutrophication in the last century, *D. galeata* was able to invade and establish in lakes that had been dominated by *D. longispina*, followed by extensive hybridisation and introgression. The subsequent re-oligotrophication of these lakes did not restore taxa composition and genetic architecture of species (Brede et al. 2009; Rellstab et al. 2011). In the most unproductive lake, however, the invasion failed (Rellstab et al. 2011) and experimental results showed that *D. galeata* requires eutrophic food conditions to persist (Spaak et al. 2012). In addition a large survey, conducted in 2003 and 2004 on 43 peri-alpine lakes, revealed that a high historic total phosphorus concentration, a proxy for trophic state, explained the dominance of *D. longispina x galeata* hybrids,
i.e. hybrids dominated in lakes that had undergone considerable eutrophication. The same study demonstrated that the parental species *D. galeata* mainly occured in lakes south of the Alps while *D. longispina* dominated in lakes north of the Alps where it was already dominant before eutrophication (Keller *et al.* 2008). This observations led to our working hypothesis that *D. galeata* is the original and native species in lakes in the southern peri-alpine range (see also Spaak *et al.* 2012). The third parental species *D. cucullata* has received far less attention in the above mentioned studies although it is present in peri-alpine lakes (Abbott *et al.* 2013) and known for e.g. Lake Greifensee (Guyer 1910; Gams 1922; Sandrock 2005), Lake Constance (Einsle 1987), Lake Neuenburgersee, Lake Murtensee, Lake Bielersee, Lake Sihlsee and Lake Iseo (Keller *et al.* 2008).

In the present study we aimed to reconstruct eutrophication history as well as taxa composition and patterns of hybridisation from sediments of southern-alpine lakes to test if *D. galeata* is indeed native to the southern lakes and to investigate the fate of *D. cucullata* during periods of trophic changes.

**Material and Methods**

**Study sites**

Lake Varese and Lake Endine are two natural lakes situated in Northern Italy. Lake Varese is a monomictic lake with a surface area of 14.8 km², a mean depth of 11 m and a maximum depth of 26 m. Lake Endine is a shallow, dimictic lake with a surface are of 2.13 km², a mean depth of 5.6 m and a maximum depth of 9 m. Both lakes underwent a phase of human-caused eutrophication followed by re-oligotrophication upon remediation measures (e.g. Garibaldi *et al.* 1997; Osservatorio dei Laghi Lombardi 2005; Zaccara *et al.* 2007).

The increasing trophic conditions in Lake Varese became evident in the 1950s and the lake subsequently reached a hypereutrophic state with mean annual total phosphorus (TP) concentrations around 350 µg P L⁻¹, peaking from the 1970s to the 1980s (Guilizzoni *et al.* 1986; Premazzi *et al.* 2003; Osservatorio dei Laghi Lombardi 2005; Zaccara *et al.* 2007). The severe deterioration of water quality, frequent fish kills and toxic cyanobacterial blooms led to the implementation of several remediation measures starting from the late 1980s (Rossi & Premazzi 1991; Giovannardi *et al.* 1999; Premazzi *et al.* 2003; Zaccara *et al.* 2007). Consequently, the trophic state of Lake Varese improved and reached a eutrophic state by the beginning of this century (Osservatorio dei Laghi Lombardi 2005; Zaccara *et al.* 2007).

The trophic state of Lake Endine was first investigated beginning of the 1970s and the lake was classified as eutrophic. A later study in the mid-1980s revealed the same result with mean annual phosphorus values around 50 µg P L⁻¹ and maximum peak values of 150-200 µg P L⁻¹ reached
in 1984-85. The lake's trophic state returned to mesotrophic conditions by mid of the 1990s mainly due to the installation of a sewage collection system (Mosello et al. 1991; Garibaldi et al. 1997; Osservatorio dei Laghi Lombardi 2005).

Nine sediment cores of 63 mm (Varese) and 18 cores of 62 mm (Endine) diameter, respectively, were collected with gravity corers from deep parts of the lakes (Varese: N45.830417°/E8.718278°, ca. 20 m water depth; Endine: N45.779833°/E9.940608°, 9 m water depth) during spring and summer 2010 and stored at 4 °C in the dark until processing. Cores were cut longitudinally into halves. One half core from each lake was then sliced into 1 cm slices, dated by measuring $^{137}$Cs and $^{210}$Pb activity (Appleby 2002) and used as reference core. Lake Varese sediment cores showed annual lamination (i.e. varves) and allowed to corroborate the age model by varve counting. In addition, total phosphorus (TP) content was determined in duplicates from the reference cores. Briefly, the freeze-dried sediments were subjected to peroxidisulfate oxidation (Ebina et al. 1983; Müller et al. 2007) and analysed in duplicates using a Bran+Luebbe AutoAnalyzer 3 (Norderstedt, Germany). The remaining core halves were aligned with the respective reference core based on visible sedimentological characteristics. A subset of cores that could be aligned with high certainty was selected, sliced accordingly and sediment samples were stored in petri-dishes at 4 °C in the dark until further processing. In order to avoid smear contamination 1-2 mm of the surface and the outer margins of each core half were removed.

**Ephippia preparation, hatching and DNA extraction**

Ephippia were extracted from sediments from three half cores for Lake Varese and four half cores for Lake Endine. Sediments were sieved through metal sieves of different mesh sizes (250 µm, 224 µm and 125 µm) under running tap water. Subsequently, ephippia were collected under a stereo microscope, washed with autoclaved nanopure water and counted.

Ephippia obtained from two of the four core halves from Lake Endine were incubated singly in 48-well plates containing filtered lake water (0.45 µm filter, Sartorius Stedim AG, Switzerland) at 20 °C and a light-dark cycle of 16:8 h to induce hatching. The 48-well plates were checked every other day over a period of three weeks and hatchlings were transferred to 100 mL jars and kept in culture. DNA was extracted from hatchlings after clonal reproduction as well as from hatchlings that had died before reproduction according to the hot sodium hydroxide and Tris (HotSHOT) protocol (Montero-Pau et al. 2008) using 50 µL of alkaline lysis and neutralising buffer, each.

The remaining ephippia from Lake Endine and all ephippia from Lake Varese were placed singly in drops of autoclaved nanopure water and opened with sterilized insect needles. Eggs were counted and assigned a quality value ranging from 1 (good quality) to 4 (bad quality) based on their shape and coloration. Afterwards, eggs were transferred into single 200 µL tubes containing 20 µL
alkaline lysis buffer. Then, eggs were crushed with a fresh pipette tip and DNA was extracted following the HOTShot protocol (Montero-Pau et al. 2008) using 20 μL of neutralising buffer.

In addition, we extracted DNA from a set of reference clones of each species and hybrids of the *Daphnia longispina-galeata-cucullata* species complex using 50 to 100 μL of each HotSHOT buffer, depending on the size of the individuals. These reference clones were sampled from different European populations and well-characterised via morphological identification, allozyme electrophoresis and via microsatellite analysis in this study (see below, Table S2, Figure 6f). Several of these clones have been used in previous studies and were characterised with additional methods (e.g. 12S rDNA sequencing, 16S restriction fragment length polymorphism) (e.g. Petrusek et al. 2008a; Yin et al. 2010; Rellstab et al. 2011).

**Ephippia measurements**

In the case of Lake Varese, the length of ephippia obtained from one core was measured prior to dissection. For that purpose, pictures were taken at a 1.6 fold magnification with a Moticam 2300 3.0 Megapixel camera (Motic Deutschland GmbH, Wetzlar, Germany) mounted on a Leica MZ9.5 stereo microscope (Leica Microsystems GmbH, Wetzlar, Germany). Subsequently, total length (excluding the spine) of ephippia was measured with Motic Images Plus 2.0 software (Motic Deutschland GmbH, Wetzlar, Germany).

**Microsatellite analysis**

In order to assess species composition and patterns of hybridisation we used nine polymorphic microsatellite markers (SwiD1, SwiD2, SwiD4, SwiD10, SwiD12, SwiD14, SwiD15, Dp512 and DaB10/14) that were developed for the *Daphnia longispina-galeata-cucullata* species complex (Brede et al. 2006) and successfully employed in several studies (Brede et al. 2009; Thielsch et al. 2009; Yin et al. 2010; Rellstab et al. 2011; Thielsch et al. 2012). All markers were combined into a single Multiplex-PCR. Each 11.5 μL PCR reaction consisted of 5.75 μL Multiplex PCR Master Mix (Quiagen, Hilden, Germany), 2.75 μL PCR-grade water, 1.5 μL extracted DNA and 1.5 μL primer mix containing forward and reverse primers in equimolar concentrations (SwiD1, SwiD2, SwiD4, SwiD14 and SwiD15: 0.4 μM; SwiD10 and SwiD12: 0.3 μM; Dp512: 0.5 μM; DaB10/14: 0.15 μM). All forward primers were fluorescently labelled (Table S1) and purchased from Microsynth AG (Balgach, Switzerland). DNA, extracted from reference clones and hatchlings, was diluted 1:3 in PCR-grade water prior to PCR to ensure equal conditions. PCR reactions started with 15 min initial denaturation at 95 °C, followed by 33 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 90 s, elongation at 72 °C for 60 s and a final extension step of 30 min at 60 °C. In order to check for the influence of 3’ adenylate overhangs and optimise allele binning, we repeated the Multiplex PCR for all reference clones and a subset of samples with pigtailed reverse primers (Brownstein et al. 1996).
using the same PCR conditions as described above, except for running 34 cycles. Each PCR plate included two positive controls (D. galeata clone G100, Tjeukemeer, The Netherlands; D. longispina clone H7, Lake Constance, Germany) and two negative controls containing DNA extraction buffer and PCR grade water, respectively, instead of DNA.

For fragment analysis, 1 µL of PCR product was diluted 1:8 in nanopure water. Then, 1 µL of the diluted PCR product was mixed with 8.75 µL HiDi and 0.25 µL GeneScan500 LIZ size standard (Life Technologies, Carlsbad, CA, USA) in 96-well plates and loaded on an ABI 3130XL Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) for capillary electrophoresis. Binning and scoring of microsatellite alleles was done in GeneMapper v.4 (Life Technologies, Carlsbad, CA, USA).

Temporal patterns of taxonomic composition and hybridisation were visualised with a factorial correspondence analysis (FCA) implemented in Genetix 4.05 (Belkhir et al. 1996-2004) using pooled data sets, comprising all resting egg samples, hatchlings and reference clones that were scored at a minimum of seven loci, for each lake.

Allozyme analysis

Allozyme analysis is applicable to fresh or frozen animals but not to resting eggs. Therefore, allozyme electrophoresis was performed for hatchlings from Lake Endine and reference clones in culture to obtain independent evidence for taxonomic assignment. Allozyme electrophoresis was performed for two polymorphic and species-specific enzyme loci (AO, aldehyde oxidase, enzyme commission number [EC] 1.2.3.1.; AAT, aspartate aminotransferase, EC 2.6.1.1) following Keller and Spaak (2004) and individuals were classified according to Nason and Ellstrand (1993).

Statistical analyses

Kenadall’s tau (τ) rank correlations between TP content measured from the sediments, available TP data measured from the water column (Osservatorio dei Laghi Lombardi 2005 and data provided by Leoni B. and Garibaldi L.) and ephippia density were calculated using the cor.test function in R version 3.0.1 (R Core Team 2013).

Results

We successfully reconstructed taxon composition and patterns of hybridisation of the Daphnia longispina-galeata-cucullata species complex over more than seven decades in Lake Varese and more than four decades in Lake Endine.

Age models and total phosphorus

For both lakes, $^{137}$Cs and $^{210}$Pb age models were obtained that were well in accordance. In addition, varve counts of Lake Varese cores were consistent with the isotope-based age models.
Figure 1: Ephippia distribution in the sediments of Lake Varese (based on three half cores). The histogram shows ephippia densities over time. The red line represents the TP content measured from the sediments and diamonds indicate TP concentrations measured from the water column (Osservatorio dei Laghi Lombardi 2005). (The water column TP levels measured in 1969 likely represent outliers).
Figure 2: Ephippia distribution in the sediments of Lake Endine (based on 4 half cores). The histogram shows ephippia densities over time. The red line represents the TP content measured from the sediments and diamonds indicate TP concentration measured from the water column (Osservatorio dei Laghi Lombardi 2005, Leoni B. and Garibaldi L., unpublished data).

TP content measured from the sediments was well in accordance with the course of TP concentrations reported from the water column (Kendall’s $\tau = 0.67$, $p < 0.01$, $n = 10$) and reflected the eutrophication history of Lake Varese (Figure 1). In Lake Endine, the course of the TP content in
the sediments indicated increasing trophic conditions from the 1960s (Figure 2) but did not mirror the well-documented re-oligotrophication process from the late 1980s (Garibaldi et al. 1997; Osservatorio dei Laghi Lombardi 2005, Leoni B. and Garibaldi L., unpublished data), resulting in a negative and non-significant correlation between TP content in the sediments and reported TP values in the water column (Kendall’s $\tau = -0.42$, $p > 0.08$, $n = 11$).

**Ephippia densities and size**

Ephippia density was positively correlated with proxies for trophic state in both lakes with ephippia density peaks during phases of maximum eutrophication (Figure 1 and 2). For Lake Varese, the correlation between ephippia density and sediment TP content was highly significant (Kendall’s $\tau = 0.41$, $p < 0.001$, $n = 39$), however, the correlation with available water column TP values was not significant (Kendall’s $\tau = 0.18$, $p > 0.47$, $n = 10$). Furthermore, ephippia density showed strong fluctuations in Lake Varese (Figure 1). In Lake Endine, ephippia density changed gradually over time (Figure 2) and was significantly correlated with water column TP (Kendall’s $\tau = 0.53$, $p < 0.027$, $n = 11$) but non-significantly with sediment TP content (Kendall’s $\tau = 0.19$, $p > 0.15$, $n = 30$).

The ephippia size distribution of Lake Varese revealed a steep drop in ephippia size in the second half of the 1960s and ephippia size remained at low levels until the end of the 1970s. This drop was followed by an steady increase in ephippia size over almost three decades that plateaued in the recent years (Figure 3 and 4).

**Taxonomic composition and patterns of hybridisation**

For the genetic analysis, we extracted a total of 2,346 eggs for Lake Varese and 1,675 eggs for Lake Endine. Eggs were better preserved in the sediments of Lake Varese and therefore, we obtained 608 microsatellite multi-locus genotypes (MLG) for Lake Varese but only 67 MLGs for Lake Endine, 16 of which representing hatchlings. An examination of our reference clones in the factorial correspondence analyses (FCA) revealed that all three species are well resolved by the first two axis and F1 hybrid genotypes take intermediate positions between parental species (Figure 6f). Some of the genotypes identified as F2 hybrids and backcrosses by allozyme analyses clustered together with the parental species, indicating that two-dimensional representation of our data in the FCA might not reveal the full extent of hybridisation or a miss-classification by allozymes (see also Rellstab et al. 2011). Overall, allozyme-based taxon assignments and FCA results were well in accordance. We observed species-specific null alleles for *D. cucullata* for markers SwiD12 and SwiD15, however, running the FCA excluding these two markers returned very similar results and we therefore kept the markers in the analysis.
Figure 3: Ephippia distribution and ephippia size analysis from the same sediment core of Lake Varese. The histogram shows ephippia densities and black dots indicate mean ephippia size (± SEM) over time. Histograms of ephippia size for selected time points are shown in Figure 4.
The analyses of the taxa composition in Lake Varese showed a clear succession of species accompanied by pervasive hybridisation (Figure 5). Contrary to our working hypothesis, we detected only genotypes that clustered together with *D. longispina* reference clones in the oldest sediments...
around the 1940s (Figure 5a). Until the end of the 1960s we encountered presumable *D. longispina* genotypes and an increasing number of genotypes intermediate to *D. longispina* and *D. galeata* (Figure 5a,b). By the end of the 1960s, we found evidence for the presence of *D. galeata* and the first appearance of *D. cucullata* around 1969 (Figure 5b). Subsequently, *D. cucullata* was the dominating species in the egg bank during the 1970s (Figure 5c) and we also detected a small number of genotypes intermediate to *D. cucullata* and *D. longispina*. Towards the end of the 1970s, *D. galeata* reappeared and until mid of the 1990s we observed a period with *D. galeata*, *D. cucullata* and their intermediate genotypes. During that period *D. cucullata* and hybrid resting eggs diminished and *D. galeata* started dominating the egg bank. This dominance continued until 2010, when we collected the cores, although in the recent years an increasing number of genotypes intermediate to *D. longispina* and *D. longispina*-like genotypes was found.

Although the low quality of resting eggs in Lake Endine sediments did not allow for a successful analysis of samples from sediment layers prior to the 1960s, similar results were obtained for Lake Endine (Figure 6). The oldest resting eggs analysed from the mid-1960s represented *D. galeata*. Subsequently, *D. cucullata* resting eggs were found in the late 1960s and early 1970s (Figure 6a), a period corresponding to the time of the first evidence for this species in Lake Varese (Figure 5b). The pattern of taxa succession in the following periods was also almost identical to the situation in Lake Varese. *D. galeata* resting eggs reappeared in the 1970s (Figure 6b) and replaced *D. cucullata* by the mid-1980s (Figure 6c). Also intermediate genotypes were found during this phase. *D. galeata* remained the dominating taxon in the recent years, however, some evidence for hybrids between *D. galeata* and *D. longispina* was found (Figure 6d). The allozyme analysis of hatchlings from Lake Endine supported the microsatellite data. In total, 16 allozyme genotypes were obtained, 11 individuals were assigned to *D. galeata*, 4 eggs were identified as backcrosses of *D. longispina-* *galeata* hybrids to *D. galeata* and 1 individual represented a F2 hybrid between *D. longispina* and *D. galeata*. Individuals with *D. longispina* alleles hatched from sediments dated to the end of the 1980s and in the 1990s. Furthermore, a single hatching from the mid-1960s revealed a *D. longispina* allele (backcross).
Figure 5: Temporal patterns of taxonomic composition and hybridisation of the *D. longispina-galeata-cucullata* species complex in Lake Varese. Scatterplots show the first and second axis of a factorial correspondence analysis. Percentage of explained variation is indicated in parentheses. The legend indicates time periods and selected reference clones. Sample sizes for the different time periods are given in parenthesis. Three representative reference clones for each species are plotted in panels a) - e). (Sp...*D. longispina*, cuc...*D. cucullata*, gal...*D. galeata*).
Figure 6: Temporal patterns of taxonomic composition and hybridisation of the *D. longispina-galeata-cucullata* species complex in Lake Endine. Scatterplots show the first and second axis of a factorial correspondence analysis. Percentage of explained variation is indicated in parentheses. The legend indicates time periods and reference clones. Sample sizes are given in parenthesis. Three representative reference clones for each species are also plotted in panels a) - e). In panel f) all reference clones and their allozyme taxon assignment, are shown. (B gal (lsp)...Backcross to *D. galeata*, B lsp (gal)...Backcross to *D. longispina*, F1 gal/lsp...F1 hybrid *D. galeata* and *D. longispina*, F2 gal/lsp...F2 hybrid *D. galeata* and *D. longispina*, lsp...D. longispina, cuc...D. cucullata, gal...D. galeata).
Taxonomic composition and ephippia size

A comparison of ephippia sizes with our ordination analysis showed a strong association of ephippia size with taxa clusters (Figure 7). The smallest ephippia sizes corresponded to genotypes that clustered together with *D. cucullata* reference clones and the largest ephippia sizes were measured for the *D. galeata* cluster. *D. longispina* ephippia size was intermediate, however, sample size was extremely low. The interspecific hybrids showed intermediate ephippia sizes compared to those of the parental species.

**Figure 7:** Temporal patterns of taxonomic composition and hybridisation of the *D. longispina-galeata-cucullata* species complex and corresponding ephippia size in Lake Varese. The x- and y-axis represent the first two axis of a factorial correspondence analysis (FCA) and ephippia size is plotted on the z-axis. Percentage of variation explained by the FCA is indicated in parentheses. The FCA coordinates of three representative reference clones for each species are also plotted. (lsp...*D. longispina*, cuc...*D. cucullata*, gal...*D. galeata*).
Discussion

Our study reveals drastic shifts in taxon composition and extensive hybridisation in the *D. longispina-galeata-cucullata* complex over the past decades and provides evidence that *D. longispina* is also native to lakes south of the Alps. We discuss our findings in the context of the known ecological characteristics of taxa, trophic change and shifts in fish predation regimes.

Following the approach of Rellstab *et al.* (2011), we successfully reconstructed the trophic history of Lake Varese from its sediments. The highly significant correlation of sediment TP content and available TP data measured from the water column during winter mixis (Osservatorio dei Laghi Lombardi 2005) and the excellent match with literature reports (Rossi & Premazzi 1991; Premazzi *et al.* 2003; Zaccara *et al.* 2007) suggest that sediment TP content is a suitable proxy for trophic state in Lake Varese (Figure 1). In Lake Endine, sediment TP content showed an increase starting in the 1950s and 1960s that very likely reflects the onset of eutrophication of the lake. Our TP data, however, fails to reconstruct the re-oligotrophication process (Figure 2), that is for large parts very well documented by monthly measurements of the water column TP (Garibaldi *et al.* 1997; Osservatorio dei Laghi Lombardi 2005, Leoni B. and Garibaldi L., unpublished data). A possible explanation for this discrepancy may be a considerable improvement of oxygenation of bottom water layers in the shallow and dimictic lake during re-oligotrophication, changing sediment chemistry and phosphorus transfer between sediments and water column (Garibaldi *et al.* 1997). Furthermore, the extent of the eutrophication process was much greater in Lake Varese than in Lake Endine (e.g. Osservatorio dei Laghi Lombardi 2005) and therefore it might also have resulted in a more distinct signature in the sediments.

The correlation of ephippia densities with proxies of trophic state (Figure 1 and 2) is well in accordance with other studies on the *D. longispina-galeata-cucullata* complex (Keller *et al.* 2002; Jankowski & Straile 2003; Rellstab *et al.* 2011) and probably reflects changes in population density and composition of taxa with differences in sexual investment (Jankowski & Straile 2003; Keller *et al.* 2007). The absence of statistical significance in the correlation of water column TP and ephippia density in Lake Varese is most likely an artefact due to strong fluctuations in ephippia densities in Lake Varese and low number of comparisons. The correlation with sediment-derived TP values, covering a longer time period, is indeed highly significant. The stronger fluctuations of ephippia densities in Lake Varese compared to Lake Endine may reflect more distinct shifts in taxa composition or effects of extreme conditions during peak eutrophication (i.e. lake anoxia, cyanobacterial blooms) on population density in Lake Varese. The higher values for peak ephippia densities in Lake Varese (Figure 1 and 2) are likely a consequence of higher trophic conditions and greater water depth allowing for higher population densities.
Our reconstruction of genetic patterns resolved the presence of all three parental species and interspecific hybrids. In a recent study, Thielsch et al. (2012) highlight that the selection of microsatellite markers is crucial for the detection of hybrids, due to difficulties associated with cross-species amplification of markers, i.e. species specific differences in amplification success. The fact the we found intermediate genotypes between all three parental species and a comparison with our reference clones (Figure 6f) reveals clearly that we could discriminate parental species and hybrids with our marker set. We, however, note that our ability to visualise later generation hybridisation and backcrossing may be limited with the type of analysis we used (FCA) and therefore we may have underestimated the full extent of hybridisation.

The observed shifts in taxon composition over time coincided with changes in the trophic state of both lakes. The presence of *D. longispina* resting eggs in pre-eutrophication periods in Lake Varese (Figure 5a) is in accordance with the known ability of this species to cope with low food levels (e.g. Weider 1993; Flößner 2000; Keller et al. 2008; Rollstab et al. 2011; Spaak et al. 2012). For Lake Endine we did not obtain usable DNA from the pre-eutrophication period, however, we found indirect evidence for the past presence of *D. longispina* in form of a *D. longispina* allozyme allele detected in a hatchling from the 1960s. The presence of *D. longispina* in the pre-eutrophication period rejects our working hypothesis of *D. galeata* being the native species in lakes south of the Alps (see also Keller et al. 2008; Spaak et al. 2012) and suggests that, similar to the situation north of the Alps, *D. longispina* represents the native species. This is supported by data suggesting a population expansion of *D. longispina* around the post-glacial formation of the peri-alpine lakes (Thielsch, A. unpublished data).

The increasing trophic conditions in the 1950s and 1960s (Figure 1 and 2) (Garibaldi et al. 1997; Osservatorio dei Laghi Lombardi 2005; Zaccara et al. 2007) rendered the lakes more suitable for *D. galeata*, a species preferring meso- to eutrophic conditions (Flößner 2000) and requiring higher food levels to sustain populations (Weider 1993; Spaak et al. 2012). We actually detected *D. galeata* genotypes in the sediments of both lakes during that period (Figure 5b and 6a). In Lake Varese, *D. longispina* resting eggs disappeared and the transition to *D. galeata* was preceded by the production of hybrids. This finding is consistent with a scenario of *D. galeata* gradually invading the lake. At the beginning of the invasion process, *D. galeata* represents the rare species and, in the absence of mate choice, the chance of reproducing sexually with the native and abundant *D. longispina* is much higher than with conspecifics. With increasing abundances of *D. galeata* and a decrease of *D. longispina*, conspecific matings of the invading species become more likely and *D. galeata* resting eggs appear in the egg bank (Figure 5a,b). An alternative explanation would be an invasion of hybrids into Lake Varese, e.g. via dispersal of ephippia. Such a scenario seems rather unlikely because several studies
indicate that hybrids are usually produced locally (Spaak 1997; Petrush et al. 2008b; Yin et al. 2010) and show reduced sexual fitness (Keller & Spaak 2004; Keller et al. 2008).

Our sample sizes for old sediment layers are low due to the senescence of resting eggs and therefore conclusions should be drawn carefully (Figure 5 and 6). Nevertheless, the general pattern of *D. galeata* invasions into lakes dominated by *D. longispina* upon eutrophication associated with extensive hybridisation is in perfect accordance with results of paleogenetic reconstructions in peri-alpine lakes north of the Alps (Weider et al. 1997; Jankowski & Straile 2003; Brede et al. 2009; Rellstab et al. 2011). These studies have not considered the fate of *D. cucullata*, the smallest member of this species complex, despite its known occurrence in some of those lakes (Gams 1922; Thomas 1944; Einsle 1987; Sandrock 2005). Indeed, *D. cucullata* plays a crucial role in the *Daphnia* population history in Lake Varese and Lake Endine. By the end of the 1960s, *D. cucullata* appeared in the resting egg bank of both lakes. This invasion was particularly well exemplified in Lake Varese. The first genetic evidence for *D. cucullata* was found around 1969 right at the time when *D. galeata* had presumably established (Figure 5b). The subsequent shift to *D. cucullata* was not accompanied with the presence of hybrids, indicating that the transition happened within a short time period. This is consistent with a coinciding drastic decrease in ephippia size in the sediments within short time showing a bimodal distribution of ephippia size (Figure 3 and 4). Our comparison of genetic data and ephippia size indicated that small sized ephippia are in fact attributable to *D. cucullata* (Figure 7). *D. cucullata* remained dominant in the egg bank until the end of the 1970s (Figure 5c). The appearance of a few putative *D. longispina* x *cucullata* hybrids, however, suggests that *D. longispina* was present at some point during that time, e.g. originating from the egg bank or from nearby Lake Maggiore. *D. cucullata* is also the most frequent taxon encountered in Lake Endine sediments during that time and occurs together with *D. galeata* (Figure 6a,b). It seems reasonable that the further progress of eutrophication over the 1960s and 1970s had facilitated the establishment of *D. cucullata*, known for its high food requirements (Gliwicz 1990) and preference for eutrophic conditions (e.g. Flößner 2000). Though, the complete absence of *D. galeata* during that time period, having similar preferences, in Lake Varese needs further explanation.

Possible reasons are a potential selective advantage for *D. cucullata* in the face of cyanobacterial blooms (Gliwicz & Lampert 1990), typical for highly eutrophic conditions, and/or size-selective fish predation (Spaak & Hoekstra 1997; Spaak et al. 2000; Spaak & Boersma 2006). While cyanobacterial blooms have also been observed during periods of *D. galeata* dominance (Giovannardi et al. 1999) (Figure 5) and therefore represent a less likely explanation, patterns of fish abundance suggest size-selective fish predation as the main causative factor. Lake Varese, once famous for excellent fish yields, underwent a drastic change in fish abundance and species composition in the course of eutrophication. During the initial eutrophication period, fish catches
were high and the proportion of important planktivorous fish, in particular an endemic bleak species named Arborella (*Alburnus arborella*) and Eurasian perch (*Perca fluviatilis*), increased until the early 1970s (Ceccuzzi et al. 2010) (Figure 8).

![Diagram](chart.png)

**Figure 8:** Total fish catches and Arborella (*Alburnus arborella*) catches in Lake Varese from 1957 to 1980. The arrow indicates the first appearance of *D. cucullata* in the resting egg bank. No data are available for 1963 (*`). Figure modified after CISPP (2005).

At the same time, *D. cucullata* ephippia started dominating the resting egg bank (see arrow in Figure 8). As a consequence of hypereutrophication (e.g. bottom and lake anoxia), fish catches and abundance of Arborella and Eurasian perch rapidly declined from 1973 and the more tolerant fish species rudd (*Scardinius erythropthalmus*), common carp (*Ciprinus carpio*), crucian carp (*Carassius carassius*), European catfish (*Silurus glanis*) and catfish (*Ictalurus melas*) started dominating. In line
with this reduction of planktivorous fish, genotypes intermediate to *D. galeata* and *D. cucullata* as well as *D. galeata* genotypes started to reappear in the sediments in the 1980s (Figure 5d). The following period was characterised by the co-occurrence of *D. galeata*, *D. cucullata* and their hybrids with a gradual shift towards *D. galeata* dominance in the egg bank (Figure 5d,e). In addition to the reduction of fish predation pressure, the on-setting re-oligotrophication of Lake Varese may additionally have favoured *D. galeata* over *D. cucullata* because *D. galeata* is expected to be the more efficient food competitor (Gliwicz 1990). This steady transition over several years is also evident from the slow increase in mean ephippia size until the millennium (Figure 3 and 4).

Again a similar pattern of taxonomic change was observed for Lake Endine (Figure 6b,c,d). Unfortunately, we do not have reliable fish data for this lake, however, the present fish community is similar to Lake Varese, suggesting that the same processes (i.e. changes in fish predation and food quantity) might have been at work. The last *D. cucullata* genotype in the Lake Endine sediments was found around 1984 while the species appeared in the resting egg bank of Lake Varese until 1996. This could be due to the overall higher extent of eutrophication in Lake Varese resulting in a time lag between Lake Endine and Lake Varese during re-oligotrophication (Figure 1 and 2). In the most recent period, *D. galeata* resting eggs were still the most abundant fraction in the resting egg bank but hybrids between *D. galeata* and *D. longispina* as well as *D. longispina*-like genotypes reappeared (Figure 5e and 6d). This is consistent with the findings of a field survey in 2003 and 2004 by Keller et al. (2008) and an analysis of the pelagial *Daphnia* community in Lake Endine in 2010/2011 (unpublished data), reporting mainly *D. galeata* and a smaller proportion of hybrids in the two lakes. This pattern suggests that re-oligotrophication measures have restored the trophic conditions to an extent that allows for the persistence of *D. longispina* or at least *D. galeata x longispina* hybrids again.

A dominance of *D. galeata* and hybrids during eutrophic conditions and an increase of hybrids and *D. longispina* upon re-oligotrophication is also observed in several peri-alpine lakes north of the Alps (Brede et al. 2009; Rellstab et al. 2011) suggesting that this is a more general pattern during periods of trophic changes. Moreover, population genetics analysis propose a recent population expansion of *D. galeata* coinciding with a period of eutrophication during the last century in many European lakes (Thielsch, A. unpublished data). The most obvious difference between lakes lies in the fact that hybrids in the northern lakes represented *D. galeata x longispina* hybrids whereas mainly *D. galeata x cucullata* hybrids were found in the analysed lakes south of the Alps. A comparison of paleogenetic reconstructions of lakes north and south of the Alps (Brede et al. 2009; Rellstab et al. 2011, this study) may suggest that *D. cucullata* is more important in the lakes analysed in this study. General differences in fish predation regimes or temperature between lakes at different sides of the Alps could be invoked as plausible explanations for this observation. An alternative and
more likely explanation lies in the fact that our selection of lakes has probably influenced this result. First, the lakes we analysed are rather small and shallow compared to the investigated lakes north of the Alps and are therefore more prone to exhibit considerable trophic changes and high fish predation pressure. Second, in the sediments of Lake Greifensee, the lake most comparable to Lake Varese with respect to size and eutrophication history, considerable numbers of *D. cucullata* resting eggs are in fact found during the eutrophication period (Sandrock 2005). Third, Keller *et al.* (2008) observed *D. cucullata* more frequently in lakes north of the Alps.

*D. longispina* was virtually absent from the resting egg bank during eutrophication which was not generally the case for lakes in the North (Brede *et al.* 2009; Rellstab *et al.* 2011). The absence of *D. longispina* resting eggs may be due to high fish predation, competitive exclusion by *D. galeata* or an extreme reduction in sexual reproduction in response to eutrophication (see Jankowski & Straile 2003). A recent study by Spaak *et al.* (2012) revealed that eutrophic food conditions do not only allow for the persistence of *D. galeata* but are also favourable for *D. longispina*. The evidence for higher vulnerability to fish predation compared to *D. galeata* is circumstantial (see Spaak *et al.* 2012) but appears to be the most likely explanation in the moment. In addition, the formation of anoxic water layers during periods of eutrophication might especially interfere with the predator avoidance strategy of *D. longispina*, i.e. diurnal vertical migration (Stich & Lampert 1981), particularly in shallow lakes.

Altogether, our study demonstrates, that the taxonomic composition of the *D. longispina-galeata-cucullata* species complex shifted drastically during a phase of human-caused eutrophication and re-oligotrophication in two peri-alpine lakes south of the European Western Alps. The observed taxonomic shifts are well in agreement with the known ecological characteristics of species and mainly explained by differences in food conditions and size selective fish predation. This temporal taxonomic succession is remarkably well in accordance with the spatial distribution of these taxa along gradients of fish predation and food quantity in canyon-shaped reservoirs (Seda *et al.* 2007; Petrusek *et al.* 2008b), underpinning the role of ecological factors for taxonomic composition.

The succession of taxa was accompanied by extensive hybridisation between all three parental species. Similar patterns are also reported for lakes north of the Alps with evidence for pervasive introgression and irreversible changes of the genetic architecture of *Daphnia* species (Brede *et al.* 2009; Rellstab *et al.* 2011). Despite of frequent hybridisation, in particular during periods of trophic change, the *D. longispina-galeata-cucullata* species complex has not yet collapsed into a hybrid swarm and undergone speciation reversal as it happened to several whitefish species in peri-alpine lakes during eutrophication (Vonlanthen *et al.* 2012). The fact that the parental *Daphnia* species are still genetically distinct is probably attributable to stronger reproductive isolation (e.g.
low sexual fitness of hybrids), higher dispersal capacities and thereby the chance for parental species to persist in undisturbed habitats and the presence of a reservoir of ancient genotypes, i.e. the resting egg bank.

Hybridisation is not only a threat to the genetic integrity of species but can also constitute a chance for the acquirement of new adaptive alleles via adaptive introgression (Pardo-Diaz et al. 2012; Abbott et al. 2013). The extensive hybridisation in the *D. longispina-galeata-cucullata* species complex in the last decades and the important role of ecological factors suggest a high potential for adaptive introgression and require further research. New sequencing technologies in combination with well-characterised genetic archives, as describe in this study, represent a powerful toolbox to investigate hybridisation, introgression and adaptation over time (Orsini et al. 2013).

In conclusion, we provide evidence for significant changes in taxonomic composition and frequent hybridisation in the *D. longispina-galeata-cucullata* species complex during a phase of trophic change. We suppose fish predation pressure and food quantity and quality as driving factors for taxonomic shifts. Furthermore, we highlight the role of the so far neglected *D. cucullata* for the evolutionary history of this species complex in peri-alpine lakes. Moreover, we add valuable biogeographical data by showing that *D. longispina* was presumably native also to the peri-alpine lakes south of the Alps prior to eutrophication. Our study contributes to the understanding of the effects of anthropogenic environmental changes on the evolutionary ecology of a key planktonic grazer and presents new model systems for future research.

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**References**


Gams H (1922) *Naturschichte der Gemeinde Maur* Pfarrer G. Kuhn, Mauer Switzerland


Chapter 5  Eutrophication and hybridisation


Eutrophication and hybridisation


**Supplementary Information**

**Table S1**: Summary information on microsatellite markers. Primer sequences (pigtail sequence in parentheses), final concentration in the Multiplex PCR reaction and fluorescent labels of forward primers are given.

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**Table S2**: Information on reference clones and hatchlings from Lake Endine (LEH) used in factorial correspondence analysis (FCA). All reference clones and hatchlings have been characterised based on allozyme analysis and morphological identification at minimum. Allozyme taxon assignment and information on the origin of the reference clones are given. The three reference clones of each species that are plotted in Figure 5a) – f) and 6a) – e) are indicated in bold. (B gal (isp)...Backcross to D. galeata, B isp (gal)...Backcross to D. longispina, F1 gal/isp...F1 hybrid D. galeata and D. longispina, F2 gal/isp...F2 hybrid D. galeata and D. longispina, isp...D. longispina, cuc...D. cucullata, gal...D. galeata)

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Chapter 6: Conclusions and Outlook

The overall aim of this thesis was to investigate the effects of human-made environmental change on the ecology, evolution and biogeography of two major Daphnia species complexes. The focus was laid on the consequences of eutrophication, micropollutants and climate change for these aquatic keystone species. In Chapters 2 to 5, I present evidence for biological invasions, hybridisation and species extinction associated with environmental changes in past decades and identified potential threats due to on-going changes. However, the study of the impact of environmental change on biota requires sound knowledge of the distribution of species. Thus, this thesis also contributes important biogeographic information on past and present distributional patterns. Underlying ecological factors were identified and will be useful for predictions on potential future species ranges and extinction risks.

Cultural eutrophication of aquatic systems has raised a lot of attention worldwide. In particular, due to the fact that eutrophication has also noticeable consequences for humans, e.g. algal blooms, fish kills and water quality issues (Correll 1998; Schindler 2006; Smith & Schindler 2009; Schindler 2012). However, eutrophication has also less obvious impacts on aquatic ecosystems, such as reduction in species diversity, changes in species composition, invasions, introgressive hybridisation and speciation reversal (Seehausen et al. 1997; Correll 1998; Seehausen et al. 2008; Brede et al. 2009; Smith & Schindler 2009; Vonlanthen et al. 2012). The implementation of successful remediation measures has led to the re-oligotrophication of many water bodies, especially in developed countries, and reversed many of the apparent effects of eutrophication. Nevertheless, eutrophication constitutes a massive issue in many developing countries (Nyenje et al. 2010; Vörösmarty et al. 2010; Pernet-Coudrier et al. 2012; Schindler 2012). Paradoxically, initiatives have been launched in Central Europe that promote to artificially increase the trophic state in several re-oligotrophied lakes with very low nutrient levels again. These considerations are motivated by the expectation of higher fish yields in more nutrient-enriched lakes and totally fade out the long-term consequences of trophic changes for species and ecosystems. Indeed, Chapters 2 and 5 provide evidence for an impact of eutrophication on the composition and evolution of species that has not been reversed by the successful reduction of nutrient loads. The most obvious pattern emerging from the reconstruction of Daphnia populations in peri-alpine lakes was the invasion and establishment of Daphnia species during periods of trophic change. Simberloff et al. (2013) have only recently called for more attention as to how anthropogenic changes of ecosystems may facilitate invasions. The findings presented in Chapter 2 and 5 suggest together with previous work (Weider et
al. 1997; Jankowski & Straile 2003; Chase & Knight 2006; Brede et al. 2009; Rellstab et al. 2011; Kokocinski & Soininen 2012; Spaak et al. 2012) that human-caused ecosystem changes play a significant role in biological invasions. Another important result was the observation that the effects of eutrophication on the lake biota could not simply be reversed by re-oligotrophication. For instance, European *D. pulicaria* was still present in Lower Lake Constance and even started to expand its distributional range after restoration of the lake's trophic state (Chapter 2). Furthermore, the *Daphnia* populations of Lake Varese and Lake Endine, situated south of the European Western Alps, still harbour the genetic legacy of past invasions and subsequent hybridisation events (Chapter 5). This is in line with results reported from lakes north of the Alps (Brede et al. 2009; Rellstab et al. 2011). The pervasive introgressive hybridisation and the storage of invasive species in the resting egg bank render it highly questionable if the original *Daphnia* populations in peri-alpine lakes can ever be restored again. In addition, manipulations of the trophic state of re-oligotrophied lakes would on one hand render conditions suitable again for invasive taxa which could easily re-colonise the water column from the resting egg bank and on the other hand open a door for new invaders (see also Rellstab et al. 2011).

A major goal in Chapter 2 and 5 was also to reconstruct the genetic processes at work during successful invasions. For the invasion of *D. pulicaria* into Lower Lake Constance (Chapter 2) we were able to successfully combine available historic information (Einsle 1987) and the results of a genetic analysis of a well-preserved resting egg bank. In particular the initial phases of a biological invasion into large water bodies are difficult to capture. Therefore, the situation in Lower Lake Constance represented an exceptional opportunity to reconstruct the full course of an invasion – from the first colonisers to the establishment of the invasive species. The genetic patterns we found are consistent with a scenario of multiple introductions from source populations pre-adapted to high trophic conditions and subsequent monopolisation (Sakai et al. 2001; De Meester et al. 2002; Kolbe et al. 2004; Frankham 2005; Kelly et al. 2006; Roman & Darling 2007). The specific life history characteristics of *Daphnia*, e.g. the ability to reproduce clonally and the production of resting stages, have very likely contributed to the success of this invasion.

In our study on the *D. longispina-galeata-cucullata* species complex in two peri-alpine lakes south of the Alps (Chapter 5), we found extensive hybridisation between species as a consequence of the invasion of non-native species. Our findings support and complement previous studies conducted on lakes north of the Alps (Weider et al. 1997; Jankowski & Straile 2003; Brede et al. 2009; Rellstab et al. 2011; Spaak et al. 2012) that revealed an invasion of *D. galeata* into lakes that underwent eutrophication and subsequent introgressive hybridisation with the native *D. longispina*. In addition we highlighted some new aspects. On one hand, we provided an answer to the long-standing question which species is native to the peri-alpine lakes south of the Alps (e.g. Keller et al. 2008;
Spaak et al. 2012) by demonstrating the presence of *D. longispina* in sediment layers of the pre-eutrophication period. On the other hand, we found evidence that *D. cucullata* has played a much more important role in peri-alpine lakes than has been recognised so far. Although *D. cucullata* is also reported for peri-alpine lakes north of the Alps (Sandrock 2005; Keller et al. 2008), it has not received much attention in these lakes and its significance for the evolutionary history of the *D. longispina* species complex has likely been considerably underestimated (but see e.g. Spaak & Hoekstra 1995, 1997). Our data on Lake Varese revealed a detailed picture of as to how different ecological factors affected by eutrophication and oligotrophication, particularly food quantity and quality and size-selective fish predation pressure, shape the taxonomic composition and patterns of hybridisation in this species complex. Unique for this study is the succession of all three species (*D. longispina, D. galeata* and *D. cucullata*) and their hybrids in this lake.

The results of Chapter 2 and 5 are both based on the analysis of *Daphnia* resting egg banks (Brendonck & De Meester 2003). Criticism has been raised that the resting egg bank might not always reliably reflect the past species composition in the short-term, in particular the relative contribution of taxa (Jankowski & Straile 2003; Keller & Spaak 2004). On the contrary, several studies demonstrate that the resting egg bank is suitable to reconstruct the fate of evolutionary successful lineages (sensu Brede et al. 2009) over the long-term (e.g. Cousyn et al. 2001; Brede et al. 2009; Rellstab et al. 2011). A comparison of our data with available historic information on *Daphnia* populations in the investigated lakes revealed a very good fit and suggested that the long-term patterns of taxonomic and evolutionary change are well reflected in the lake sediments.

Orsini et al. (2013) have recently highlighted the potential of resting egg banks in combination with new genomic tools (Colbourne et al. 2011) for the study of evolution. I propose that Lower Lake Constance and Lake Varese harbour biological archives that are highly appropriate for such studies. In both lakes a high number of well-preserved resting eggs can be found over a time period of drastic ecological changes. Moreover, the history of the lakes and the *Daphnia* populations is well-documented (e.g. Chapter 2 and 5). Therefore, these lakes are predestined for the study of adaptation (European *D. pulicaria* in Lower Lake Constance) and adaptive introgression (*D. longispina* complex in Lake Varese) on the genomic level.

After developed countries had introduced governmental regulations and installed sewage treatment plants to control eutrophication, a new threat to aquatic systems became apparent. For our daily life we produce and use thousands of natural and synthetic chemicals, e.g. in the form of personal care products, pharmaceuticals, detergents, pesticides, and industrial chemicals. As with essentially every human product, these substances are released to the environment and enter natural waters via e.g. urban and industrial sewage, surface runoff, spray drift and leaching from
agricultural areas. These so-called micropollutants are typically found in low concentrations, however, many of them are known to be of toxicological relevance and they are usually present in complex mixtures making them an environmental risk that is extremely difficult to predict (Schwarzenbach et al. 2006; Sumpter 2009).

In Chapter 3 we investigated the effect of a complex mixture of organic contaminants on the fitness of the resting egg bank of the *D. longispina* species complex and revealed significant effects on hatching success and mortality. We chose our study setup for several reasons. First, dormant stages are of high relevance for many aquatic invertebrates and determine population dynamics, dispersal capacities and evolutionary potential (for reviews see Hairston & Kearns 2002; Brendonck & De Meester 2003; Gyllstrom & Hansson 2004). Second, micropollutants are known to accumulate in lake sediments (Zennegg et al. 2007; Kohler et al. 2008; Chiaia-Hernandez et al. 2012). Third, resting eggs can stay in the sediments over decades before hatching which may considerably prolong exposure time. Furthermore, our setup was exceptional because we exposed ephippia produced by a natural population of the *D. longispina* species complex in Lake Greifensee to a mixture of contaminants that was actually measured in the sediments of the same lake (Chiaia-Hernandez et al. 2013). In my opinion, this environmentally realistic combination of study organism and exposure substances is of immense importance for risk assessment, however, it is not necessarily common practice in ecotoxicological studies. Prior to their approval, many substances are tested for their aquatic toxicology using *D. magna* as a test organism (OECD 2004, 2012). This approach poses several limitations for risk assessment. First of all, species- and even genotype-specific differences in the susceptibility towards pollutants are known for *Daphnia* (e.g. Buser 2011). Second, *D. magna* is a typical pond species while the *D. longispina* species complex is often found in large lakes (Flößner 2000) and, therefore, they are very likely exposed to different pollution scenarios.

Toxicological studies on resting stages are very scarce. Nevertheless, Chapter 3 and a recent study by Nais et al. (2013) suggest a significant impact of micropollutants on resting egg banks with far-reaching consequences for benthic-pelagic coupling and evolutionary potential of *Daphnia* spp. and highlight the urgent need for further studies on this topic. In addition, we developed and described a method that allows for convenient handling and testing of large amounts of resting eggs that are required in experiments with ephippia from natural populations.

Chapter 4 represented an important contribution to the taxonomy and biogeographical knowledge of two members of the *Daphnia longispina* species complex. This complex is exceptionally well-studied for Central and Northern Europe and North America. Nevertheless, information from the rest of Europe and in particular Asia is scarce, in part due to the inaccessibility of many areas, and
new lineages have only recently been described from these regions (Ishida & Taylor 2007; Petrush & al. 2012; Zuykova & al. 2013).

*D. dentifera* Forbes, 1891 and *D. longispina* O. F. Müller, 1776, are both known to inhabit oligotrophic water bodies, and in this study we found them in lakes in the Himalaya and Pamir mountains, respectively. For the Himalaya, a "pale" *Daphnia* has already been described in previous work but was misclassified as *D. longispina* or *D. longispina* var. *aspina* Vereschagin, 1911 (e.g. Brehm & Woltereck 1939; Löfler 1969; Manca & al. 1994, 1998). Our molecular analysis clearly identified the species inhabiting the Himalayan lakes as *D. dentifera*, providing the first evidence for the occurrence of the species on the Eurasian mainland, and the morph sampled in the Pamir mountains was assigned to *D. longispina*, previously unknown for this area. We also proposed an ecological model explaining the occurrence of *D. dentifera* in the lakes in the Himalayan mountains and identified water depth and turbidity, proxies for habitat stability and UV stress, respectively, and total phosphorus, an indicator for trophic conditions, as significant explanatory variables.

Obviously, comprehensive knowledge of the distribution of species and their ecological niche are prerequisite for the investigation and predictions of the effects of environmental change on the biota. Based on our data on *D. dentifera* in the Himalaya we identified a potential threat to this population imposed by climate change. Most of the lakes are connected to glacial drainage systems and the rapidly progressing retreat of glaciers in the Himalaya (Cruz & al. 2007) is likely to affect the hydrology of these lakes. Consequences for the UV refuge, determined by water depth and turbidity, and nutrient loads and thus for the distribution and survival of *D. dentifera* are to be expected.

In conclusion, this thesis provides multiple lines of evidence for a major impact of human caused environmental change on the distribution, ecology and evolution of hybridising *Daphnia* species complexes. Moreover, fundamental data for decision makers are provided that should be considered in current debates on eutrophication control in developing as well as developed countries, the implementation of water-treatment technologies and governmental regulations to prevent the release of micropollutants into the environment and measures to slow down climate change. Furthermore, potential model systems for future studies of adaptation, adaptive introgression (Chapter 2 and 5), ecotoxicological studies using resting eggs from natural populations (Chapter 3) and for studies on the effects of rapid climate change (Chapter 4) were presented.

**Outstanding questions and recommendations for future research**

**Chapter 2:** In the sediments of Lower Lake Constance we encountered a completely homozygous genotype, that dominated the resting egg bank for several years during the first phases of the invasion. We interpreted this genotype as a product of strong selfing, however, we could not rule out alternative scenarios, e.g. the presence of an obligate parthenogenetic clone. A targeted study of this
Conclusions and Outlook

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genotype, e.g. with a higher number of markers and additional hatching experiments, could shed light on the role of the reproductive mode for the invasion success of European *D. pulicaria*. Furthermore, we found evidence for a recent expansion of European *D. pulicaria* into Lake Greifensee. I recommend a population genetic monitoring of this invasion as it represents the opportunity to follow the invasion process in real-time and allows for a higher temporal resolution. A comparison of the invasion in the pelagial and its fingerprint in the lake sediments would provide useful information on how reliably the resting egg bank reflects the genetics of an invasion in the pelagial. However, it also remains to be seen if the species will successfully establish in Lake Greifensee. Moreover, a survey of additional peri-alpine lakes could reveal if the range expansion of European *D. pulicaria* represents a local phenomenon or a more general pattern.

The egg bank of European *D. pulicaria* in Lower Lake Constance is very well-preserved and covers a period of ecological change, i.e. the full duration of lake re-oligotrophication. In addition, an array of molecular tools has recently been developed for a closely related member of the *D. pulex* species complex (Colbourne et al. 2011). In line with the suggestions of Orsini et al. (2013), I recommend to take this opportunity and to combine experimental studies of hatchlings from different time periods with genome-wide scans to study adaptation to oligotrophic conditions.

In Chapter 2, I also analysed several samples from alpine lakes in Austria, Switzerland and Italy. In mitochondrial phylogenies they are clearly positioned within the European *D. pulicaria*, however, several microsatellite markers revealed unusual patterns, e.g. indications for the presence of triploid loci, and these populations are ecologically clearly differentiated from lowland populations in Central Europe. Polyploids of hybrid origin are indeed known for the *D. pulex* species complex and preferentially found in harsh environments (e.g. Colbourne et al. 1998). Preliminary results from sediment cores sampled in the Italian Alps suggest that they were already present and presumably more abundant several hundred years ago. In general, these high-alpine populations have not received much attention and I therefore suggest to put more effort into the study of phylogenetics and ecology of these populations right on our doorstep.

Chapter 3: Here, we showed an effect of a mixture of micropollutants detected in lake sediments on hatching success of resting eggs and hatchling mortality. Follow-up studies on the effects of single substances and different combinations at different concentrations are now needed. The identification of the relevant chemicals or mixtures and concentrations is required for serious risk assessment. I also recommend to extent our approach to additional lakes and species relying on a resting egg bank. The effects we found should also be further explored in mechanistic studies to clarify the mode of action of the respective chemicals.
Chapter 4: Our findings in Chapter 4 and recent studies (Ishida & Taylor 2007; Petrush et al. 2012; Zuykova et al. 2013) highlight the need for field surveys and phylogenetic studies in Eastern Europe, Asia and alpine habitats. Apparently, a considerable fraction of the diversity in the genus *Daphnia* is still undiscovered and our knowledge on their biogeography is not as extensive as previously thought.

Furthermore, I propose an integrated monitoring of *D. dentifera* populations and lake ecosystems in the Khumbu region (Himalaya) to investigate effects of climate change on aquatic key species and food webs. Apparently, climate change progresses at an exceptionally high rate in this area (Cruz et al. 2007) and the ecological factors explaining the occurrence of *D. dentifera* are closely linked to climatic factors. This combination suggests this system as a useful model to study effects of climate change on the species and ecosystem level and the Pyramid International Laboratory in the Khumbu area provides the necessary infrastructure.

Chapter 5: Our investigations of the resting egg bank of Lake Varese together with results from lakes north of the European Western Alps (e.g. Brede et al. 2009; Rellstab et al. 2011) unravel a high potential for the study of genomic consequences of hybridisation and potential adaptive introgression in the *D. longispina-galeata-cucullata* complex. In order to achieve this goal, I propose a concerted action of several labs working on this complex to develop the required genomic toolbox (e.g. reference genomes) and to characterise relevant adaptive traits and their underlying genetics. Time for launching such a project is short as the senescence of resting eggs makes it more and more difficult to obtain well-preserved *D. longispina* DNA from the pre-eutrophication period.

The past ecological changes have resulted in increased admixture and introgression in the *D. longispina* complex. Therefore, well-characterised reference clones of the parental species are a valuable resource. Here, I propose that a set of reference clones covering the genetic variation within each parental species should be characterised with available marker systems, and these clones should be maintained and used by all labs working on the complex. Such a common effort would minimise work load and ensure comparability of results.

More research is also needed to clarify why *D. longispina* resting eggs actually disappeared from the resting egg banks with increasing trophic conditions. Spaak et al. (2012) showed that *D. longispina* also benefits from higher food levels. There is some evidence that differences in the vulnerability to fish predation and different predator avoidance strategies might be involved (see Spaak et al. 2012), however, more direct evidence from experiments would be appreciable. In addition, the impact of invertebrate predation on the succession of species during periods of trophic change is unexplored. Large-scale mesocosm experiments (e.g. Spaak & Boersma 2006) or experimental ponds would allow for the manipulation of food quality and quantity, predation levels
and temperature. Such a setup would be useful to test single and combinatorial effects of candidate ecological factors on different taxa combinations and could help to elucidate the role and relative importance of the different ecological factors for the composition of *Daphnia* communities in more detail.

Another open question that remains to be answered is the origin of the *D. galeata* and *D. cucullata* that invaded into the peri-alpine lakes. Population genetic comparison of the earliest *D. galeata* and *D. cucullata* genotypes encountered in the sediments of peri-alpine lakes with recent European and Asian populations as well as paleogenetic reconstructions of potential source populations outside of the Alpine range could help to answer the question if the invaders originated from different sources or a common source population or area, respectively.

References


Conclusions and Outlook


Curriculum Vitae

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Education

10/2009-present
Ph.D. studies in Aquatic Ecology, Dept. of Environmental Systems Science, ETH Zurich / Dept. of Aquatic Ecology, Eawag
Supervisor: Piet Spaak

05/2009-09/2009
Lab and field assistant with Peter Eklöv and Richard Svanbäck, Department of Ecology and Evolution / Limnology, Uppsala University

Master studies in Zoology, University of Innsbruck (with honors). Supervisor: Bernd Pelster

Bachelor studies in Biology, University of Innsbruck (with honors)

06/1999
School leaving examination (with honors)
Further education

- **03/2013** Genetics and Genomics of Speciation Workshop (Frontiers in Speciation), Organisers: Ole Seehausen, Roger Butlin, Luzern (CH)
- **06/2012** Summer School in Bioinformatics and Population Genomics. Organisers: SIB Swiss Institute of Bioinformatics and Doctoral Program in Population Genomics, Adelboden (CH)
- **04/2012** Genetic Diversity Analysis course. Genetic Diversity Center (GDC), ETH Zurich (CH)
- **02/2012** Winter School in Landscape Genetics. Organisers: Rolf Holderegger, Felix Gugerli, Janine Bolliger, WSL Birmensdorf (CH)
- **10/2011** Hybridisation and Speciation workshop (Frontiers in Speciation). Organisers: Roger Butlin, Mike Ritchie, Jacek Szymura, Gregynog Hall (UK)
- **06/2010** Guarda workshop in Evolutionary Biology. Faculty: Tim Clutton-Brock, Sarah P. Otto, Dieter Ebert, Sebastian Bonhoeffer, Guarda (CH)
- **05/2007** Practical course in Electrofishing. Institute for Water Ecology, Fisheries and Lake Research, Federal Agency of Water Management, Scharfling (AUT)
- **03/2007** Eawag PEAK course on fish ecology and identification "Fische in Schweizer Gewässern". Organisers: Rudolf Müller, Armin Peter, Kastanienbaum (CH)
- **09/2005** Practical course in marine ecology with Ragnhild Asmus at the Wadden Sea Station of the Alfred Wegener Institute in Sylt (GER)
- **08/2004** Practical course in aquatic ecology with Hans Güde at the Department of Hydrobiology, Landesanstalt für Umwelt, Messungen und Naturschutz Baden-Württemberg, Langenargen (GER)

Meetings, Symposia, and Seminars

- Symposium of Ecology and Evolution Doctoral Students (SeeDS), Bern (CH), 17 December 2012. Oral presentation
- Zurich Interaction Seminar, Zurich (CH), 8 October 2012. Oral presentation
- Second ECO-PhD Symposium, Dübendorf (CH), 22 May 2012. Oral presentation and member of organizing committee
- Fifth International Limnogeological Congress (ILIC), Constance (GER), 31 August - 3 September 2011. Oral and poster presentation
- First ECO-PhD Symposium, Dübendorf (CH), 9 May 2011. Oral presentation and member of organizing committee
- Sixth International Symposium on Eco-Evolutionary Dynamics, Leuven (BE), 3-5 February 2011. Poster presentation
- The role of Littoral Processes in Lake Ecology Symposium, Hegne (GER), 29-31 January 2010
- Basel Interaction Seminar, Basel (CH), 15 Dezember 2010. Oral presentation
- Swiss Russian Seminar: Cladocerans (Crustacea) as model organisms for ecological and evolutionary research: approaching the age of genomics, Fribourg (CH), 8-10 November 2010. Oral presentation
- Resurrection Ecology Symposium, Herzberg (CH), 14-17 September 2009

Project collaborations and jobs

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<th>Year</th>
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<tr>
<td>2007-2009</td>
<td>Freelancer at the environmental consulting agency &quot;ARGE Limnologie - angewandte Gewässerökologie GesmbH&quot; (fish population surveys within the scope of the EU Water Framework Directive)</td>
</tr>
<tr>
<td>2004-2009</td>
<td>Freelancer at the environmental consulting agency &quot;ARGE für Fisch- und Gewässerökologie&quot; (fish population surveys and fish relocations)</td>
</tr>
<tr>
<td>2003-2008</td>
<td>Project collaborator in the Interreg III A project &quot;Trout Exam-Invest&quot; (identification of autochthonous trout lineages, electrofishing, fish breeding, stocking and population monitoring)</td>
</tr>
<tr>
<td>2005-2007</td>
<td>Project collaborator in the Interreg III A project &quot;Der Piburger See - Untersuchung der Wasserqualität und des Fischbestandes zur Optimierung des Seenmanagements&quot; (fish population survey and management)</td>
</tr>
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Teaching assistance

- "Evolutionary Biology: Laboratory Course" (701-1416-00L) with Piet Spaak, ETH Zurich
- "Ecology, Aquatic and Terrestrial Ecosystems" (551-0712-00L) with Otto Seppälä, ETH Zurich
- "Marinbiologischer Kurs Madeira" (a field course in marine biology) with Bernd Pelster and Reinhold Hanel, University of Innsbruck
- "Baupläne im Tierreich" (a course in animal anatomy) with Ruben Sommaruga and Rudolf Hofer, University of Innsbruck
- "Labormethoden" (a course in basic lab methods) with Reinhard Lackner and Rudolf Hofer, University of Innsbruck
- "Tierphysiologische Übungen I" (a course in animal physiology-electrophysiology) with Hans Moser and Thorsten Schwerte, University of Innsbruck
Abbott Livia, Languages Sarah O. Hudson, Technology Chiaia Evolutionary
• "Tierphysiologische Übungen II" (a course in animal physiology-metabolism) with Reinhard Lackner, University of Innsbruck
• "Tierphysiologische Projektstudie" (an advanced course in animal physiology) with Margit Egg, University of Innsbruck
• "Physiologisches Spezialpraktikum: Adultes Herzkreislaufsystem" (a course in the physiology of the circulatory system) with Thorsten Schwerte, University of Innsbruck

Co-supervised students

Sarah Oexle, Master Thesis, University of Constance
Sarah Wolf, Bachelor Thesis, ETH Zurich
Livia Baumgartner, Bachelor Thesis, ZHAW Zurich

Further skills

Languages | German, English
Licenses | Boat driving license, Diving license CMAS 1*

Publications


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