Long-term genetic shifts in a microcrustacean egg bank associated with anthropogenic changes in the Lake Constance ecosystem

LAWRENCE J. WEIDER^{1*}, WINFRIED LAMPERT¹, MARTIN WESSELS², JOHN K. COLBOURNE³ AND PETRA LIMBURG¹

¹Max-Planck-Institut für Limnologie, Postfach 165, D-24302 Plön, Germany

²Limnologisches Institut, Universität Konstanz, Postfach 5560, D-78434 Konstanz, Germany

³Department of Zoology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

SUMMARY

Diapausing egg banks of aquatic zooplankton have the potential to remain viable for decades or even centuries, and can thus harbour potentially high levels of genetic variation. Diapausing (ephippial) eggs from the *Daphnia galeata-hyalina* complex (Crustacea: Anomopoda) in Lake Constance (Bodensee), Germany, were isolated from sections of dated sediment cores, hatched in the laboratory, and established as a clone bank. We used cellulose acetate electrophoresis at four polymorphic enzyme loci (*Pgm*, *Pgi*, *Ao*, and *Got*) to examine long-term temporal changes in the genetic composition of the hatchling pool. Our results indicate that significant shifts have occurred in the genetic structure of this population, which parallel concomitant shifts in the trophic state of Lake Constance during the past 25–35 years. Here we discuss the utility of egg bank propagules as good model organisms to study microevolution, as related to past environmental change.

1. INTRODUCTION

The factors influencing the maintenance of genetic variation in natural populations have been the subject of considerable theoretical (Hedrick *et al.* 1976; Hedrick 1986, 1995), as well as empirical work (Endler 1986). Much work has focused on the importance of spatial environmental heterogeneity on maintaining genetic polymorphisms (see Hedrick (1986) for review), while temporal environmental factors have not been examined to nearly the same extent (Templeton & Levin 1979; Hairston *et al.* 1996; Hedrick 1995). In fact, within the field of theoretical population genetics, the role of temporal environmental heterogeneity in maintaining genetic variation in populations has been viewed as limited (Hedrick 1986).

A feature of many organisms, particularly plants and many invertebrates, is the presence of long-lived life history stages such as dormant seeds (Leck *et al.* 1989; McGraw 1993), or diapausing eggs (Hairston *et al.* 1995), which allow an organism to 'escape in time' from harsh environmental conditions. In addition, these dormant stages play a key role in what has been termed the 'storage effect' (Chesson 1983); i.e. long-term influence of diapausing propagules on either population or community biodiversity. As pointed out

by Hairston *et al.* (1996), historically published reviews in the field of population genetics have not taken the potential importance of the 'storage effect' into account, and only recently have more general population genetics models examined this feature in any detail (Ellner & Hairston 1994; Hedrick 1995).

Among aquatic invertebrates, members of the Cladocera (Crustacea: Anomopoda), and in particular, species in the genus *Daphnia* are well-known for producing diapausing eggs (encased in a resistant structure termed an ephippium), that function both as a dispersal (i.e. spatial 'escape'), as well as a dormant (i.e. temporal 'escape') life-history stage deposited in the sediments of many freshwater bodies. The hatching of long-lived resting eggs of freshwater zooplankton (i.e. decades or even centuries old) has been reported for the cladoceran, *Ceriodaphnia pulchella* (14-year-old) by Moritz (1987), while successful hatching of decades-old eggs of copepods, cladocerans, and rotifers (ranging in age from 5–40 years), has been reported by Marcus *et al.* (1994). A recent study by Hairston *et al.* (1995) reported the hatching of resting eggs of the calanoid copepod, *Diaptomus sanguineus*, that were dated at an astounding 330 years old. As promoted by Hairston *et al.* (1995), the impact of long-lived life cycle stages like dormant eggs of zooplankton on population and community-level processes has been under-appreciated and overlooked by many researchers.

*Author for correspondence (wei@mpil-ploen.mpg.de).

The focus of our study was to examine the population genetic structure of the ephippial egg bank of the *Daphnia galeata-hyalina* complex in Lake Constance (Germany) by hatching ephippial eggs from different sediment horizons (ages), establishing a clone bank, and then using allozyme electrophoresis to assess changes in the genetic composition of the population through time. Only one previous study (Carvalho & Wolf 1989; Wolf & Carvalho 1989) has attempted to examine the population genetic structure of the ephippial egg bank of *Daphnia*. However, any conclusions about long-term temporal changes in population genetic structure were hampered by extremely small sample sizes (Carvalho & Wolf 1989; Wolf & Carvalho 1989), and the lack of any sediment age data. To our knowledge, this present study represents the first report ever of long-term (i.e. decades-long) shifts in the population genetic structure of a zooplankter, assessed via its diapausing egg bank. We discuss our results in light of the environmental changes that have occurred in the Lake Constance ecosystem during the past 25–35 years, and we illustrate how egg bank propagules may allow one to examine the influence of past environmental changes on microevolutionary processes in natural populations.

2. METHODS

(a) Field collection and sediment dating

Sediment cores were collected in 1996 and 1997 from two deep water (>100 m deep) stations in the Obersee (Upper Basin) of Lake Constance; the 1996 core was collected 1.5–2.0 km east of station 1 (Wessels 1995, p. 27), while the 1997 core was taken from station 2 (Wessels 1995, p. 27). Parallel cores were dated using either the Zn-concentration (for the 1996 station) or lamination counting method (for the 1997 station) of Wessels *et al.* (1995). The Zn-concentration method is a high-resolution technique that can be used to date 20th century sediments with great accuracy based on interpolation between fixed reference points in the sediments. For Lake Constance, the maximum Zn concentration invariably occurs at a sediment layer dated from 1963/1964 (Wessels 1995; Wessels *et al.* 1995); we used this layer as a reference point. In addition, we calibrated the Zn-concentration method with ¹³⁷Cs-dating, another interpolation technique (Wessels 1995), and we obtained very similar results (M. Wessels, unpublished data). In contrast, the lamination counting method is a much more accurate method of dating sediments. A precise calibration between the two dating methods is somewhat difficult, especially given the typical differences in sedimentation rates at different sites in Lake Constance. However, Wessels *et al.* (1995) have compared the two dating methods, and have found the results to be highly concordant.

(b) Sediment slicing and ephippial hatching

Cores were sliced into 1 cm thick sections (sediment depths/ages in the remainder of the paper refer to the middle of each section; i.e. 0.5 cm for the 0–1 cm section), sections were placed in air-tight petri dishes, and kept at 4 °C in the dark until they were processed. To avoid the possible inadvertent transfer of ephippia between different layers during the slicing process, the outer edge of each section was sliced away with a plastic ring (3.0 cm in diameter for the 3.5 cm diameter 1996 core; 5.5 cm in diameter for the 6.0 cm

diameter 1997 core). The remaining sediments were then filtered through a series of nested metal sieves (160–350 µm mesh); ephippia were collected and transferred to clean Petri dishes containing filtered (0.45 µm) lake water, and placed in a walk-in environmental chamber, maintained at 10–12 °C and constant light (conditions that have been known to break diapause in *Daphnia*; Weider & Hebert 1987). Ephippia were monitored daily for a period of six weeks. Newly hatched daphnids were removed, carefully transferred individually to separate culture jars, and raised as clonal cultures. A total of 189 hatchlings were obtained from the 1996 core (0.5, 2.5, 3.5, 4.5, 5.5, 6.5 cm depths; the 1.5 cm depth was not examined), while 1214 hatchlings were collected from the 1997 core (0.5–10.5 cm, 13.5 and 17.5 cm depths).

At the conclusion of the hatching period, numbers of unhatched healthy-looking eggs (i.e. dark-green colour with intact membrane) were enumerated and added to the number of hatchlings to provide total viable ephippial egg densities per sediment depth. Hatching success (%) was calculated as the proportion of the total viable ephippial egg densities that actually hatched.

(c) Allozyme electrophoresis and statistical analyses

Individual clones were screened for electrophoretic variation using cellulose acetate electrophoresis (Hebert & Beaton 1989). Four polymorphic enzyme loci were screened: aldehyde oxidase (AO, enzyme commission number (EC) 1.2.3.1), glutamate-oxaloacetate transaminase (GOT, EC 2.6.1.1), phosphoglucose isomerase (PGI, EC 5.3.1.9), and phosphoglucomutase (PGM, EC 5.4.2.1). Based on previous work (Wolf & Mort 1986), which has shown that GOT is diagnostic for distinguishing between *D. galeata* (fixed for the *F*, fast allele) and *D. hyalina* (fixed for the *S*, slow allele), the vast majority of hatchlings (i.e. all 151 surviving hatchlings from the 1996 core and 988 out of 997 surviving hatchlings from the 1997 core) were *FF* homozygotes at *Got*, and therefore were classified as *D. galeata*. The remaining nine hatchlings (three from 3.5 cm, one from 7.5 cm, five from 13.5 cm) from the 1997 core were *SF* heterozygotes at *Got*, thus, indicating that they were *D. galeata* × *hyalina* hybrids. Since *Got* was nearly fixed for the *F* allele, we will not deal with it any further in this paper; we will focus the rest of our analysis and discussion on the other three loci.

Allele and multi-locus genotype frequencies were analysed using BIOSYS (Swofford & Selander 1989), while a comparison of similarity of three-locus genotypic (clonal) arrays between various sediment depths was performed via a Mantel test (Mantel 1967) using the SIMQUAL (Jaccard's index) and MXCOMP subroutines of NTSYS (Rohlf 1990).

3. RESULTS AND DISCUSSION

Dating of the cores revealed a lower sedimentation rate for the 1996 core when compared with the 1997 core. Absolute dates (sediment age in years) are given for each sediment layer in each core (table 1). Ephippial hatching success (figure 1) varied between the 1996 and 1997 cores, as well as between the various sediment layers. For the 1996 core, we observed greater than 50% hatching success down to a sediment age of 19 years (figure 1a), while for the 1997 core, we found greater than 75% hatching success down to a sediment age of 20 years (figure 1b). Decreasing hatching success with increasing sediment depth (age) has been reported

Table 1. *Allele frequencies of Daphnia ephippial hatchlings and age of sediment layers from the 1996 and 1997 sediment cores* (n = sample size for the allozyme analysis. Relative mobilities of alleles from the origin are labelled: S, slow; M, medium; M⁻, slightly slower than medium; F, fast; F⁺, super fast.)

depth (cm)	age (yr)	<i>n</i>	<i>P_{gm}</i>				<i>P_{gi}</i>		Ao				
			S	M	F	F ⁺	M	F	S	M ⁻	M	F	F ⁺
1996 core													
0.5	4.0	51	0.029	0.373	0.520	0.078	0.910	0.090	—	—	0.320	0.480	0.200
2.5	11.8	30	0.047	0.359	0.500	0.094	0.887	0.113	—	—	0.288	0.481	0.231
3.5	15.3	31	0.113	0.323	0.484	0.081	1.000	—	—	—	0.241	0.648	0.111
4.5	19.0	14	0.179	0.214	0.536	0.071	1.000	—	—	0.071	0.107	0.751	0.071
5.5	22.0	18	0.194	0.083	0.472	0.250	1.000	—	—	—	0.111	0.694	0.194
6.5	24.8	7	0.071	0.286	0.571	0.071	1.000	—	—	—	0.071	0.786	0.143
1997 core													
0.5	0	37	0.042	0.625	0.306	0.028	0.784	0.216	—	—	0.162	0.824	0.014
1.5	2.0	31	0.048	0.339	0.565	0.048	0.887	0.113	—	—	0.210	0.677	0.113
2.5	4.0	138	0.016	0.424	0.512	0.048	0.913	0.087	—	—	0.225	0.743	0.033
3.5	6.0	44	0.068	0.364	0.523	0.045	0.898	0.102	—	—	0.250	0.750	—
4.5	8.0	46	0.152	0.196	0.609	0.043	0.891	0.109	—	0.022	0.217	0.739	0.022
5.5	10.0	196	0.108	0.256	0.549	0.087	0.944	0.056	—	—	0.151	0.796	0.054
6.5	11.0	89	0.051	0.426	0.472	0.051	0.989	0.011	—	—	0.208	0.792	—
7.5	13.0	95	0.100	0.326	0.479	0.095	0.995	0.005	—	—	0.242	0.716	0.042
8.5	15.0	37	0.054	0.270	0.622	0.054	1.000	—	—	—	0.203	0.797	—
9.5	18.0	153	0.105	0.258	0.542	0.095	1.000	—	—	—	0.181	0.740	0.079
10.5	20.0	108	0.126	0.332	0.481	0.061	0.991	0.009	—	—	0.168	0.804	0.028
13.5	26.0	20	0.000	0.316	0.658	0.026	0.975	0.025	0.025	—	0.125	0.800	0.050

by various investigators (Marcus *et al.* 1994; Hairston *et al.* 1995), and may reflect decreased viability of eggs that reflects their natural longevity in the sediments, as well as, possibly being related to the length of time that eggs have been exposed to anoxic or toxic (H₂S) sediment conditions. In addition, differences in the abundance of ephippial eggs between sediment layers were also observed (figure 1). For the 1996 core, the surficial (four-year-old, 0–1 cm) sediment layer had the highest density of eggs, with a dramatic decrease in abundance in the older sediment layers (figure 1a). In contrast, the peak abundance of eggs in the 1997 core was found at intermediate depths (10- and 18-year-old layers; figure 1b).

Hatching of ephippia at a given sediment depth was well-synchronized, with distinct hatching peaks. Given the well-synchronized hatching phenology, no significant genotypic differences in time of hatching were observed. There was a significant positive correlation between sediment (egg) age (X) and the days until peak hatch (Y); i.e. older eggs take longer to break diapause (regression for the 1996 data: $Y = 6.017 + 0.247X$, $r^2 = 0.804$, $p < 0.015$; regression for the 1997 data: $Y = 7.587 + 0.376X$, $r^2 = 0.861$, $p < 0.0001$). Interestingly, similar results have been reported recently for the brine shrimp, *Artemia franciscana* (Clegg 1997) and the copepod, *Skistodiaptomus pallidus* (Dowell 1997).

Allelic arrays were very similar between the 1996 and 1997 cores (table 1); a total of five alleles at the *Ao* locus, four alleles at the *Pgm* locus, and two alleles at the *Pgi* locus were detected. Generally, genotypic frequencies were in Hardy–Weinberg (H.–W.)

equilibrium (i.e. 18 out of 18 comparisons at the *Pgm* locus, 10 out of 11 comparisons at *Pgi*), the exception being *Ao*, where only 8 out of 18 comparisons did not show significant deviations from H.–W. expected genotypic frequencies. (The ten deviant cases at *Ao* were all heterozygote deficiencies.) These data indicate that generally, the ephippial hatchling pool approximates that of a truly sexual population, at least at the *Pgm* and *Pgi* loci. These results are quite typical for large-lake populations of *Daphnia* (Mort & Wolf 1986), including the Lake Constance *Daphnia* assemblage (Weider & Stich 1992).

Results of the Mantel test, which compared similarity of clonal arrays between different sediment layers in the 1997 core, revealed a significant negative relationship ($r = -0.580$, $t = 3.359$, 250 random permutations, $p < 0.004$); i.e. as differences in age between sediment layers increases, the genetic similarity of clonal arrays between these layers decreases. These data indicate that microevolutionary changes have indeed occurred in the sediment egg bank. (Due to small samples in the deeper sediment layers of the 1996 core, we were unable to perform a similar analysis for this core.)

Significant shifts in the allelic composition of the population were observed at all loci (table 2), most noticeably at *Pgi* (table 1). We observed a dramatic decrease in the frequency of the *M* (medium) allele with a concomitant increase in the frequency of the *F* allele, which relates to approximately 15 years ago, marking the beginning of the 1980s (figure 2a). This period directly followed the peak period of eutrophication in Lake Constance during the late 1970s as

Figure 1. Hatching success (%) of Lake Constance *Daphnia galeata* for the 1996 (a) and 1997 (b) sediment cores. Density of ephippial eggs (no. of eggs $\times 10^4$ m 2) in each sediment (age) layer are shown.

exemplified by total phosphorus levels in the water column (figure 2a). A significant correlation was found between frequency changes in the *M* allele at *Pgi* and changes in total phosphorus concentration (measured using standard methods; Güde *et al.* 1998) during the past 25–35 years ($r = 0.823$, $p < 0.00003$). Similarly, a significant (albeit weaker) correlation was detected between frequency changes in the *S* allele at *Pgm* and changes in total phosphorus concentration (figure 2b;

Table 2. Results of the row $\times$ column tests of independence (*G*-tests) of allele frequency differences at *Pgm*, *Pgi*, and *Ao* for the *Daphnia* hatchlings from the different depths (cm) for the 1996 and 1997 sediment cores

locus	<i>G</i> -test	d.f.	<i>p</i>
1996 core			
<i>Pgm</i>	31.024	15	<0.01
<i>Pgi</i>	23.258	5	<0.001
<i>Ao</i>	29.780	15	<0.025
1997 core			
<i>Pgm</i>	258.754	33	<0.001
<i>Pgi</i>	247.854	11	<0.001
<i>Ao</i>	214.760	44	<0.001

$r = 0.576$, $p < 0.012$). These data suggest very strongly that changes in the genetic structure of the *D. galeata* population are directly related to anthropogenic (trophic state) changes in Lake Constance. Such trophic state changes (i.e. eutrophication, oligotrophication) are the direct result of changes in the quantity of human effluent emptying into the lake during the past 40 years. Since the implementation and diversion of effluent into tertiary sewage treatment plants began in the early 1980s, Lake Constance has experienced a dramatic decrease in total phosphorus (figure 2), along with subsequent shifts in aquatic community structure (see Güde *et al.* (1998) and Bäuerle & Gaedke (1998), for a recent compilation and summary of these changes).

Historically, characterization of the daphnid fauna in Lake Constance has been difficult (Einsle 1978, 1983a,b, 1988; Kiefer & Muckle 1959; Muckle & Dillmann-Vogel 1976; Weider & Stich 1992). Up until the early 1950s, *D. hyalina* was the only recognized daphnid species in the lake. However, Kiefer & Muckle (1959) observed a *D. galeata*-‘like’ form becoming more prevalent in Lake Constance during the 1950s. As pointed out by Einsle (1978, 1983a,b), morphological intermediates between *D. hyalina* and this *D. galeata*-‘like’ form became common in the lake during the 1960s, and a small *Daphnia* type increased in abundance starting around 1970 in the eastern (Bregenz Bay) section of the lake. Weider & Stich (1992) used allozyme electrophoresis, and categorized the daphnid fauna in Lake Constance as consisting of an epilimnetic *D. galeata* population that did not undergo vertical migration, while *D. hyalina* and *D. galeata* $\times$ *hyalina* hybrid populations that were found in deeper waters during the day, migrated into the warmer epilimnion at night.

Our allozyme data suggest that the *Daphnia* sediment egg bank in Lake Constance is dominated by ephippia produced by *D. galeata*. Only nine of the 1148 hatchlings (0.8%) did not show the characteristic FF homozygous pattern at *Got*. Quite surprisingly, we did not find a single SS homozygote, which would indicate *D. hyalina*. Either all *D. hyalina* ephippia had hatched from the sediments prior to our collecting them, or *D. hyalina* does not produce viable ephippia, an observation that is hard to reconcile, given that *D. hyalina* in

Figure 2. (a) Changes in the frequency of the M allele at *Pgi* with changes in sediment age (depth). (b) Changes in the frequency of the S allele at *Pgm* in relation to changes in sediment age (depth). Allele frequency data taken from water column samples in 1989–1990 (Weider & Stich 1992) are plotted for comparison. Changes in total phosphorus concentration (total P, $\mu\text{g l}^{-1}$) in the water column are also plotted (Güde *et al.* 1998).

Lake Constance are known to produce ephippia (contributing up to 4% of the adult population at certain times; Stich 1989). Alternatively, the fraction of ephippia that did not hatch could have contained some *D. hyalina* eggs. Given the extremely high hatching success (figure 1), especially for the 1997 core, it is once again difficult to believe that we would not detect a single *D. hyalina* ephippium in a total sample size of greater than 1100 hatchlings. Furthermore, allele frequency data (figure 2a,b) taken from water column

samples in 1989–1990 (Weider & Stich 1992) are quite similar to the allele frequencies found in the hatchling pool (from the 1989–1990 sediment horizon) in this present study, and thus do not indicate differential hatching success in the ephippial egg bank. Another alternative explanation is that *D. hyalina* ephippia may be positively buoyant (i.e. float), and so end up in shallower (<100 m deep) water away from our coring sites. We have no data to support this notion, but it is known that the external surface morphology of ephippia (i.e. the presence of projections or protrusions like spines), which could affect their buoyancy, varies among *Daphnia* species (Hebert 1995).

Previous empirical work on copepods (Hairston & De Stasio 1988) and plants (McGraw 1993) has demonstrated that sufficient genetic variation is present in egg/seed banks to allow natural populations to respond readily to changing selection pressures (e.g. predation; Hairston & De Stasio 1988). Furthermore, it is well-known that sufficient genetic (clonal) variation exists in natural *Daphnia* populations (e.g. Weider 1985) to allow a response to changing food levels. The allozyme markers used in this present study are most likely neutral (or quasi-neutral), and therefore, are probably not the direct targets of selection. Our data suggest that microevolutionary processes (e.g. selection, genetic drift, gene flow), have influenced the genetic composition of the ephippial egg bank. One might argue that genetic drift is likely to play a significant role in influencing the genetic composition of the egg bank, but this is hard to imagine given the tremendously large population sizes (i.e. ranging from $3\text{--}14 \times 10^4$ eggs m^{-2} , figure 1). In addition, gene flow cannot be entirely excluded as a factor influencing the genetic composition of the ephippial egg bank, especially given the historical observations of apparent daphnid 'species' invasions during the past 40–50 years (Einsle 1978; 1983a,b), and given the fact that Lake Constance is an 'open' system (i.e. the Rhine River flows into and out of it).

Regardless of the relative importance of these various microevolutionary factors, we are still lacking a direct link between shifts in the genetic structure of the egg bank and particular environmental parameters. We plan on bridging this gap in our knowledge by investigating the ecological response of egg bank hatchlings from various depths (ages) to different environmental conditions (i.e. food levels mimicking changes in lake trophic status) to ascertain whether such a direct link indeed exists. Results from this and subsequent studies should allow us to evaluate the use of long-dormant zooplankton propagules as good model organisms to examine microevolutionary processes, especially related to past environmental changes in aquatic systems.

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