A millennial-scale chronicle of evolutionary responses to cultural eutrophication in Daphnia

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INTRODUCTION

Understanding evolutionary responses to anthropogenic ecological change is a central challenge in contemporary biology (Kareiva et al. 1992; Hoffmann & Sgro 2011). This challenge has been approached using two distinct strategies: long-term experimentation with extant populations (Harmon et al. 2009; Bell & Gonzalez 2011), and reconstruction of the genetic structure of past populations (Bidle et al. 2007; Härnström et al. 2011). While manipulative experiments are useful, resulting data on metazoans often span only a few generations. On the other hand, studies reconstructing past population genetic structure often lack phenotypic data. Resurrection ecology provides a powerful tool to address this issue by allowing the integration of genetic data from resting eggs with phenotypic observations on individuals revived from dormant stages (Hairston et al. 1999; Decaestecker et al. 2007).

While much of our knowledge on the evolutionary responses to environmental change is related to the global carbon cycle and temperature (Parmesan 2006), global environmental change includes drastic changes to biogeochemical cycles of other biologically relevant elements such as nitrogen (N) and phosphorus (P) as well (Schlesinger 1997). In fact, the advent of artificial fertilisers has played a pivotal role in human population growth, leading to profound environmental alterations (Smil 2000). Notably, while the first artificial P fertiliser was patented in 1859 (Brown 2000), 2 days before the publication of the Origin of Species, the evolutionary consequences of human alterations to the P cycle remain poorly understood.

Direct observation of evolutionary change through time, complemented by long-term environmental data, will allow us to validate our current understanding of mechanisms of selection based on empirical data from spatial studies (Orsini et al. 2013). Although we lack access to historical genetic material in many organisms, a variety of microbes, plants and animals provide natural genetic archives of past populations via dormant propagule banks (i.e. seeds, eggs, cysts (Evans & Dennehy 2005)) found in soil, permafrost/ice and lacustrine/marine sediments.

In addition to historical DNA archives, lake sediments harbour long-term records of water chemistry that can be reconstructed using paleolimnological methods (Engstrom & Wright 1984), including phosphorus (P) loading, a key driver of eutrophication (i.e. nutrient enrichment of waterbodies (Schindler et al. 2008)). A common effect of excessive P-loading is the increase in biomass and P-content of seston (i.e. planktonic living and non-living matter), altering the nutrition of zooplankton, such as the keystone aquatic herbivore, Daphnia (Sterner & Elser 2002). Daphnia are P-rich consumers, more efficient in utilising P than other zooplankton taxa, and thus are highly sensitive to alterations in P loading (Elser et al. 2009; Hoffmann & Sgro 2011). This challenge 2 days before the initiation of industrialised agriculture in the catchment area. Population genetic structure, analysed using DNA from dormant eggs of the keystone aquatic herbivore, Daphnia pulicaria, suggested no change for c. 1500 years prior to striking shifts associated with anthropogenic environmental alterations. Furthermore, phenotypic assays on the oldest resurrected metazoan genotypes (potentially as old as c. 700 years) indicate significant shifts in phosphorus utilisation rates compared to younger genotypes. Younger genotypes show steeper reaction norms with high growth under high phosphorus (P), and low growth under low P, while ‘ancient’ genotypes show flat reaction norms, yet higher growth efficiency under low P. Using this resurrection ecology approach, environmental, genetic and phenotypic data spanning pre- and post-industrialised agricultural eras clearly reveal the evolutionary consequences of anthropogenic environmental change.
et al. 1988). *Daphnia* populations harbour substantial genetic variation in sensitivity to P availability (Jeyasingh & Weider 2007; Jeyasingh et al. 2009), including the differential expression of thousands of genes (Jeyasingh et al. 2011). Most daphniids are cyclical parthenogens with mainly asexual reproduction and a sexual phase, typically at the end of the growing season, which results in the production of sexual (dormant) eggs encased in a sclerotised structure (ephippium). Ephippia remain in a dormant state, resting on the lake floor, until favourable conditions occur for hatching.

Here, we utilise paleolimnological methods (Engstrom & Wright 1984) to reconstruct c. 1600 years of environmental and population genetic history of a *Daphnia pulicaria* Forbes; 1893 population in a natural lake (South Center Lake – SC; Chisago County, Minnesota) impacted by agricultural activities and urbanisation during the past c. 150 years, thereby contrasting the genetic responses during long periods of environmental stability with those during rapid environmental change. We evaluate changes in population genetic structure in response to cultural (human-induced) eutrophication and identify loci associated with environmental change. We hypothesised that the initiation of industrialised agriculture and other anthropogenic activities in the catchment area of SC Lake c. 120 years ago would increase P loading into the lake and cause shifts in several environmental parameters known to be associated with eutrophication. We hypothesised such anthropogenic environmental shifts to coincide with shifts in allelic frequencies in this *Daphnia* population, potentially indicating altered selection leading to an evolutionary response. Furthermore, we explored whether shifts in allelic frequencies could be driven by variation in key phenotypes known to respond to P availability. Characterising the P use physiology and growth response in individuals resurrected from sediments could provide insight into the genetic basis for P use and allow for the identification of key traits in younger genotypes.

**MATERIALS AND METHODS**

**Field sampling**

**Study site**

South Center Lake (SC), Chisago County, Minnesota, is one of 24 lakes in the Minnesota Sentinel Lakes Program (MSLP), administered by the Minnesota Pollution Control Agency in conjunction with the Minnesota Department of Natural Resources, where long-term monitoring of eutrophication has been on-going (Minnesota Pollution Control Agency 2012).

**Sediment cores**

Duplicate or triplicate sediment cores were taken at c. 24 m water depth of SC (45°22.645′ N, 92°49.215′ W) in both July 2010 and July 2011, using a 1.5 m (6.93 cm diameter) single-drive Griffith sediment corer with Livingstone drive rods from the deck of an anchored pontoon boat. Negative pressure exerted by the piston corer held the core in place until it could be brought to the surface, where it was carefully placed on an extruder for on-board processing. Cores were examined for the presence of laminated (varved) sediments and absence of gas bubbles to ensure the integrity of the sediment layers. Any cores that showed possible disruption/mixing of sediments were not processed and were later discarded. One core was sliced into 1 cm sections for 210Pb dating and loss-on-ignition assays (see supplemental material for details) and another in 4 cm sections for isolation of ephippial eggs and quantification of sediment phosphorus concentrations (see supplemental material). Care was taken to prevent possible contamination of ephippial eggs between sediment layers by carefully extruding sections, slicing, and thoroughly washing extruder/slicer between samples. Samples were placed individually in 125-mL polycarbonate sample cups with lids, and placed immediately in coolers containing ice packs, and then returned to the laboratory for processing.

The signal of 210Pb sediment dating (Table S1) allowed for determination of the approximate age/sediment depth of pre-European/post-European settlement to coincide with 46 cm (ca. 1804). Below this sediment layer, the 210Pb dating method did not provide sufficient resolution. Therefore, dating of sediments below 46 cm was conservatively estimated based on the sedimentation rate at 46 cm (i.e. 0.0063 g cm⁻² year⁻¹), which represents the oldest sedimentation rate verifiable by the 210Pb dating method. (Note: sedimentation rate estimates in Table S1 appear to ‘stabilise’ at c. 44–46 cm, where values ranged from 0.0054 to 0.0064 g cm⁻² year⁻¹. This represents the early European colonisation period of c. 1855–1804.) We believe this is a reasonable, conservative approach to estimate sedimentation rates, since compression of deeper sediments is a common feature of lake sediment cores (Engstrom et al. 2009; also see Table S1), meaning that these deeper layers may be even older than this estimate. If for some reason the sedimentation rates were greater in these deeper layers, these layers could potentially be younger than our estimates. Future sediment dating that utilises Carbon (14C) methods would be required to provide more accurate dating estimates for these deeper layers.

**Molecular analysis**

**DNA extraction and amplification**

Individual eggs were separated from ephippia sampled from the sediment (details in Supplementary material). DNA was extracted from eggs (*n* = 298) using a modified HotShot protocol (Montero-Pau et al. 2008) and additionally, in the most recent layers, from ephippial hatchlings (*n* = 96) raised as clonal cultures (CTAB method (Hillis et al. 1990) for a total of 394 individuals. Genotypes were characterised using 17 microsatellite loci chosen to maximise the number of loci that could be genotyped together, without regard to their physical location or potential proximity to functional genes. Coincidently, these loci were distributed across the entire genome (Table S1). Microsatellite loci ((Colbourne et al. 2004), Table S1) were amplified in single, 25 μL multiplex reactions (Type-it PCR kit, Qiagen Inc, Valencia, CA, USA), using a MJ Research PTC-200 thermocycler with thermal cycle conditions recommended by Qiagen. Amplified microsatellites were genotyped on an Applied Biosystems 3130xl genetic analyser.

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Table 1 Results of MatSAM analysis for the detection or environmental association of allele frequencies for 15 polymorphic microsatellite loci (mapped to the D. pulex genome)

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chromosome</th>
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<th>End</th>
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The two monomorphic loci are included for completeness. Chromosome number, scaffold, and their beginning and ending position on the scaffold are shown. Four microsatellite loci could not be mapped to the genome (n/a) because the scaffolds on which they are located are not yet mapped to the genome (marked with *). Monomorphic loci are italicised. Detailed MatSAM results are shown in Table S6.

Population genetic structure

The temporal population genetic structure of D. pulex was reconstructed using 17 microsatellite loci using a model-based clustering method in a Bayesian framework implemented in STRUCTURE 2.3.3 (Pritchard et al. 2000) (Fig. 1). (15 of the 17 loci were polymorphic for the SC population. The presence of monomorphic loci is irrelevant to this analysis, because STRUCTURE relies on allele frequency differences between populations to infer ancestry of individuals.) Individuals or eggs from each sediment layer (Table S2) were defined as members of a temporal subpopulation. All samples used for DNA extraction resulted from sexual reproduction and therefore represent unique genotypes, fulfilling the requirements of statistical models that assume sexual reproduction. We computed posterior likelihood values for $K = 1$ to $K = 10$ the number of ancestral genetic clusters from which the analysed individuals are assumed to be descended. The applied model assumes admixture between populations and correlated allele frequencies, with prior information on population identity (the LOCPRIOR model (Hubisz et al. 2009). For each $K$, we ran 10 simulations with a burn-in of 50 000 Markov-Chain-Monte-Carlo (MCMC) iterations and 100 000 iterations after burn-in. The most likely value for $K$ was estimated from the greatest rate of change in the likelihood function ($\Delta K$) of successive $K$ values (Evanno et al. 2005), using the online software STRUCTURE HARVESTER (Earl & von Holt 2011) (Fig. S3). The obtained data for 10 successive runs for each of three values of $K$ including the one identified by STRUCTURE HARVESTER as the most probable were merged using CLUMP 1.1.2 (Jakobsson & Rosenberg 2007) and graphed with DISTRUCT 1.1 (Rosenberg 2004).

We examined the relationship of ancestry coefficients generated for temporal subpopulations ($K = 2$), human population census, and sediment P using Spearman Rank correlations, and tested these with a permutation test (Monte Carlo permutation test with 9999 random replicates) using PAST (Hammer et al. 2001).

Mantel tests

Partial Mantel tests were applied to remove spurious correlations due to the temporal nature of the study by controlling for temporal distance. We tested the relationship between genetic and physical or environmental distance in the sediment core. To construct distance matrices, we used genetic distance (Rousset’s (Rousset 1997) distance measurement with null-allele-adjusted $F_{ST}$, $(F_{ST}^{EAF}/(1 – F_{ST}^{EAF}))$, and environmental distances (depth (physical distance), total (ortho-)phosphorus, per cent organic matter of the sediment and human population census). Partial Mantel tests were performed with ZT (Bonnet & Van de Peer 2002). A one-tailed test was used to test the assumption that genetic distance increases with temporal or environmental distance. We applied Bonferroni correction for multiple comparisons (adjusted significance level of $P = 0.002$).

Detection of loci with environmental associations

We used MatSAM vs.2 (Joost et al. 2007), to search for environmental associations of the 15 polymorphic microsatellite loci (Table 1). The program performs multiple simultaneous univariate logistic regressions by which allele frequencies (presence/absence of any particular allele) are regressed against environmental factors to detect associations between environmental factors and allele frequencies. The significance of the logistic regression coefficients are evaluated by testing whether a model with environmental variables is more informative than the null model (the logistic regression model with a constant only), using the likelihood ratio (G) and Wald tests (Joost et al. 2007). The G-test only provides results if there are no missing data, while the Wald test does not have this limitation. We therefore ran the complete data set ($n = 394$ individuals), which included some isolates with sporadic missing data at certain loci, and performed only a Wald test. We then repeated the analysis with a subset ($n = 246$) of individuals that had no missing values at any loci in order to perform both a Wald test and G-test (Table S4).

Phenotypic assays

We measured the retention rate for phosphorus (RE), phosphorus use efficiency (PUE), and growth rate (GR) of genotypes resurrected from four sediment layers: three genotypes were tested from the 4–8 cm depth (2008–2002 AD); three genotypes from the 20–24 cm depth (1977–1967 AD); one genotype from the 52–56 cm depth (1646–1536 AD); and two genotypes from the 60–64 cm depth (1418–1301 AD). Daphnia were fed with Scenedesmus acutus algae, grown in phosphorus limiting conditions (LP; 5 $\mu$mol L$^{-1}$) and/or P replete conditions (HP; 50 $\mu$mol L$^{-1}$) at 1 mg C L$^{-1}$. For all three experiments, third clutch granddaughters of each genotype reared in
Retention efficiency (RE)
We followed established methods to quantify P retention in daphniids (DeMott et al. 1998). One-day-old Daphnia from each of the 9 test genotypes were starved for 2 h to evacuate gut contents. Eighteen individuals of each genotype were placed in 50-mL jars containing radiolabelled LP algae (details for radiolabelling in supplementary material), while another 18 individuals were placed in jars containing radiolabelled HP algae. This experiment was replicated three times. In each jar, after 10 min of pulse feeding, nine animals were immediately pipetted out from each jar, washed in N- and P-free COMBO medium (Kilham et al. 1998) and transferred to scintillation vials for estimation of radioactivity. The remaining nine animals in each jar were transferred to a new 50-mL jar containing unlabelled algae. Media was changed immediately pipetted out from each jar, washed in N- and P-free COMBO medium (Kilham et al. 1998) and transferred to scintillation vials. RE is defined as percentage of the initial radioactivity that was retained in the animal after 12 h:

\[
RE(\%) = \frac{\text{amount of radioactivity in animals after 12 h}}{\text{amount of radioactivity in animals after 10 min}} \times 100
\]

Phosphorus use efficiency (PUE)
PUE of the nine test genotypes was quantified after 5 days of experimental exposure to low P diet. PUE is defined as the amount of biomass produced per unit of somatic P concentration under P limiting conditions (Table S5). PUE = M/ Pc, where, M = dry mass (mg), Pc=P concentration of dry mass (mg). Five neonates (< 24-h old) from each genotype were grown separately in 50-mL jars and fed 1 mg C L⁻¹ day⁻¹ low P algae daily. After 5 days, the animals were dried at 60 °C for 2 days, and weighed using a microbalance (Mettler-Toledo XP2U, Columbus, OH) to the nearest µg (to determine M). Phosphorus content of the animals was estimated using sulfuric acid digestion similar to sediment analysis. This experiment was replicated five times per genotype.

Growth rate (GR)
Less than 12-h-old neonates from each of the nine genotypes were grown individually in 50-mL jars under 1 mg C L⁻¹ day⁻¹ of either HP or LP algae (Kilham et al. 1998). This experiment was replicated 10 times. Animals were gently transferred onto a microscope slide and body length (top of head to base of tail spine) was measured at the end of the experiment (day 0), and at the end of the experiment (day 5) using a Leica microscope (Leica S8APO, Leica Microsystems, IL, USA) fitted with an ocular micrometre at 4X magnification. Animals were transferred to a new 50-mL jar with fresh food daily. The observed mortality during the experiment was negligible. Growth rate is represented as the change in mm day⁻¹ (Fig. 3; Table S5).

Statistical analysis of phenotype data
We fitted nested linear mixed effects models with the ‘lme4’ package (Bates et al. 2009) for the R Statistical Software, version 3.0.3 (R Core Team 2013). Separate models were fitted for each fixed effect and their respective interaction by maximum likelihood (with the ‘REML = FALSE’ argument in the model specification) and compared against a null model that excluded all fixed effects using a likelihood ratio test (ANOVA). All models included the fixed factors ‘genotype age’ (four age groups), ‘P treatment’ (the latter only for RE and GR, with the two levels ‘high P’ and ‘low P’), and the random factor ‘genotype’ (9 genotypes), nested within ‘genotype age’. For all three experiments (PUE, GR and RE), we treated genotype age as an ordered factor and used orthogonal polynomial contrast analysis, which allows testing for a directional effect of ‘genotype age’. PUE was logit-transformed prior to analysis as recommended by Warton & Hui (2011). RE was analysed with the amount of radioactivity measured in animals after 12 h (i.e. the numerator) as the dependent variable, while using radioactivity measured after 10 min (i.e. the denominator) as a covariate.

RESULTS
Reconstructing environmental history
We quantified shifts in lake productivity parameters (organic matter and CaCO3) and the amount of phosphorus (ortho-P) in the lake sediment, over the c. 1600-year time span represented by a 96 cm sediment core from South Center Lake (SC), Chisago County, Minnesota. The percentage of organic matter remained relatively stable between 24 and 26% from the deepest layer at 96 cm (c. 314 AD), until a sediment depth of c. 35 cm (first decade of the 1900s), after which it increased to c. 34–36% in the uppermost layer, which was deposited in 2010/2011 (Fig. 1, left panel). European settlement in Chisago County began in the early mid-1800s, with a population increase from 1743 in 1860 AD to 53 887 in 2010 AD (Fig. 1, Table S1). After remaining at a constant low level for most of the previous c. 1500 years, P concentration in the sediment of SC increased in parallel to intensifying agricultural activities (ranging from 0.974 to 5.008 mg P g sediment⁻¹, Table S1), signalling the initiation of cultural eutrophication in the late-1800s (Fig. 1, Table S1). Sediment ortho-P closely tracks total farmed acreage in Chisago County (Fig. S1). Human population size was positively correlated with organic matter and sediment P (R² = 0.628 and 0.939, respectively, P < 0.01).

Temporal population genetic structure, differentiation and diversity
By extracting DNA from dormant eggs and hatchlings (total N = 394) isolated from dated sediment cores spanning c. 1600 years, we reconstructed the temporal population genetic structure of D. pulicaria from SC using 17 microsatellite loci distributed on at least 6 of the 12 chromosomes (Fig. 1, Table 1). Null allele frequencies of temporal subpopulations were low (between 0.012 and 0.057 across loci, Table S2). Two loci were monomorphic (Dp375 and Dp376), while the number of alleles ranged from 2 to 8 per locus in the remaining 15 loci across the entire dataset. Significant departures from HWE were not recorded until c. 1898 (40–44 cm), and were present in some temporal subpopulations after 1989 (Table S2). Allelic richness and private allelic richness, averaged across loci (Table
Eutrophication history and *Daphnia pulicaria* population genetic structure of the past *c.* 1600 years, in South Center Lake (Chisago County, MN). Left panel - upper *x*-axis: sediment P (green shaded zone), organic content (black line) - lower *x*-axis: human population size in Chisago County, MN (red line). Centre panel: representation of dated sediment core. Right panel: *D. pulicaria* population genetic structure, each individual represented by a horizontal bar and a black horizontal line delimits the 15 temporal subpopulations. **Note**: Due to differential amplification success between temporal subpopulations, alignment with the timeline of the left and centre panel is offset. Grey lines connect the right panel with the centre panel to guide correspondence between panels. Red arrows depict the sediment layers from which hatchlings were obtained. The indicated dates correspond to the top of each sediment layer. Detailed sediment dating in Table S1.

The Bayesian clustering analysis in *STRUCTURE* estimated *K* = 2 most likely, as determined by the Δ*K* method of *Evanno* et al. (*Evanno* et al. 2005), regardless of the admixture model applied. Results discussed here are based on the model allowing for admixture (Fig. 1). Higher numbers of *K* yielded similar information with respect to the detected transition zone and overall population genetic structure (Fig. S3), and did not provide any extra information, such as previously undetected population substructure. We detected a transition from the red cluster, which dominated the lower half of the sediment core, to the blue cluster (Fig. 1 and Fig. S3), dominating the upper core. This transition was positively correlated to human population size (Spearman *r* = 0.8299, *P* = 0.0001) and changes in sediment P (Spearman *r* = 0.625, *P* = 0.014).

Levels of temporal genetic differentiation (global *F*<sub>ST* value for SC 0.06) were low and we found no evidence for a genetic bottleneck (all *M*-values > 1, Table S2). Very low levels of genetic differentiation were observed between sequential sediment layers (*F*<sub>ST* between 0.00 and 0.04, Table S3, Fig. S5). However, patterns of isolation-by-environmental-distance were detected in partial Mantel tests, controlling for spurious correlations with temporal distance (i.e. time). These tests showed strong correlations between genetic differentiation and cultural eutrophication history: physical distance (i.e. depth, partial Mantel test, *r* = 0.6312, *P* < 0.0001), human population census (partial Mantel test, *r* = 0.6200, *P* < 0.0001), sediment P (partial Mantel test, *r* = 0.3979, *P* < 0.001),% organic matter in the sediment layers (partial Mantel test, *r* = 0.4256, *P* = 0.0039).

**Temporal patterns of allele frequencies associated with the environment**

Considering the entire data set of *c.* 1600 years, we found allelic associations at five loci that included one or more of the three measured environmental parameters (Fig. 2, Table S4). Changes in allele frequencies at these loci were most frequently associated with organic matter and sediment P. Three of these loci had associations independent of sediment age, while two loci were related to time.

**Comparison of phenotypic responses**

Phosphorus retention efficiency (RE) was significantly affected by P treatment, and was overall higher in the low P treatment. The slopes of the RE reaction norms of older genotypes in response to high and low P diets were less steep than those of the younger genotypes, as suggested by a highly significant interaction of P treatment and genotype age (Table 2, Fig. 3a). The amount of P in daphniids after 10 min did not significantly explain variation in RE retention (Table 2, parameter estimates for all phenotypic responses in Table S6).

Overall, juvenile growth rate (GR) was significantly affected by P treatment (Table 2), being lower at the low P conditions. The interaction between genotype age and P treatment was highly significant (Table 2) and showed a similar trend to the RE response with flat growth rate reaction norms for older genotypes. Younger genotypes appeared to be more strongly affected by the P-limited diet, reflected in steeper reaction norms (Fig. 3b).

Phosphorus use efficiency (PUE) was significantly explained by genotype age, indicating that younger genotypes used P less efficiently than older genotypes (Table 2, Fig. 3c).
The data presented here allowed us to reconstruct centuries of environmental history with a major period of eutrophication after European settlement in the first half of the 20th century. Although a number of studies have utilised resurrection ecology to infer adaptive responses of *Daphnia* to changes in ecological parameters (Weider et al. 1997; Hairston et al. 1999; Cousyn et al. 2001; Decaestecker et al. 2007), the time-scale of our study permitted the inclusion of data that pre-dates European settlement, and thus allows a more robust analysis of anthropogenic impacts. Prior to the present study, the oldest and most complete reconstructed population genetic profile of *Daphnia* dates back c. 200 years ago (Limburg & Weider 2002), although older (c. 700 years), more sporadic, genetic profiles exist (Murgeay et al. 2007). While these studies report genetic shifts based on dormant egg DNA, little is known about phenotypic changes associated with human impacts because the oldest resurrected daphniid egg, to date, is c. 125 years old (Caceres 1998). Here, we show changes in population genetic structure of the keystone herbivore *Daphnia pulicaria* contemporaneous with human-induced environmental change. Moreover, physiological experiments, albeit on a limited number of genotypes, indicated that resurrected genotypes estimated to be as old as c. 700 years were markedly differentially adapted in their nutrient use compared to ~decades-old descendants.

**DISCUSSION**

The data presented here allowed us to reconstruct centuries of environmental history with a major period of eutrophication after European settlement in the first half of the 20th century. Although a number of studies have utilised resurrection ecology to infer adaptive responses of *Daphnia* to changes in ecological parameters (Weider et al. 1997; Hairston et al. 1999; Cousyn et al. 2001; Decaestecker et al. 2007), the time-scale of our study permitted the inclusion of data that pre-dates European settlement, and thus allows a more robust analysis of anthropogenic impacts. Prior to the present study, the oldest and most complete reconstructed population genetic profile of *Daphnia* dates back c. 200 years ago (Limburg & Weider 2002), although older (c. 700 years), more sporadic, genetic profiles exist (Murgeay et al. 2007). While these studies report genetic shifts based on dormant egg DNA, little is known about phenotypic changes associated with human impacts because the oldest resurrected daphniid egg, to date, is c. 125 years old (Caceres 1998). Here, we show changes in population genetic structure of the keystone herbivore *Daphnia pulicaria* contemporaneous with human-induced environmental change. Moreover, physiological experiments, albeit on a limited number of genotypes, indicated that resurrected genotypes estimated to be as old as c. 700 years were markedly differentially adapted in their nutrient use compared to ~decades-old descendants.

Nutrient enrichment reflects intensified agricultural activities in the South Center watershed during European settlement...
centuries-old time span. Low levels of temporal genetic differentiation and the lack of evidence for a genetic bottleneck during the environmental transition period provided additional support that this population was connected continuously through time. The population genetic structure (Fig. 1) prior to and after a transition zone was assigned primarily to one of two distinct clusters. The shift from the ‘ancient’ to the ‘modern’ genetic cluster (i.e. the likelihood of individuals belonging to the ‘modern’ cluster) was strongly correlated with a growing human population in the SC catchment. The time-scale of this study allowed us to directly observe changes in allelic frequencies as evolutionary consequences of anthropogenic environmental change and to relate allelic frequency shifts to eutrophication parameters in 30% (i.e. 5 of 15) of the polymorphic loci. Allelic associations with the environment at these five loci, included all three measured environmental parameters (associated most frequently with organic matter and sediment P, two key indicators of eutrophication), suggesting the possibility of linkage of the microsatellite markers to loci under selection. Further characterisation of these gene-environment associations requires high resolution genomic probing (e.g. whole-genome sequencing, analysis of selective sweeps), but is currently unavailable due to the restricted amounts of high quality DNA present in diapausing eggs, especially those that are centuries old.

Concomitant to these shifts in allelic frequencies, we found striking differences between genotypes resurrected from sediments deposited centuries before and after anthropogenic perturbation in the lake's catchment. Specifically, the three physiological phenotypic traits measured revealed that: (1) c. 700-year-old genotypes retained more of the ingested P, while modern genotypes were able to regulate P retention based on P supply (Fig. 3a); (2) such differential P retention is reflected in growth rate, with modern genotypes exhibiting greater plasticity in growth compared to ancient counterparts (Fig. 3b); and finally and (3) such differences were not solely determined by somatic P content, because PUE, the measure of biomass produced per unit somatic P content under P limiting conditions varied among genotypes (Fig. 3c) and increased with age of genotype (Table 2). Together, these results indicate evolution of plasticity in key P-use phenotypes such as P retention and growth rate, although, clearly more genotypic replicates, and quantitative genetic studies are needed to provide further support for this conjecture. Previous studies on extant Daphnia report that genotypes with higher fitness in P limiting conditions had lower fitness in P replete conditions (Weider et al. 2005; Jeyasingh & Weider 2005; Jeyasingh et al. 2009). Recent work on these extant genotypes (P. Roy Chowdhury, unpubl. data) has revealed that P retention was a major factor underlying such fitness differentials, although the genotypes were not different in somatic P content. Importantly, this study found that the most P efficient genotype retained about 57% of ingested P after 12 h under low P conditions. In this study, we found that c. 700-year-old genotypes, measured under similar conditions, retained as much as 82% of ingested P after 12 h (Table S5). Such high P retention has not been observed previously in any species of adult Daphnia (DeMott et al. 1998; He & Wang 2007, 2008). Furthermore, modern Daphnia exhibit a sharp increase in P retention under P limiting conditions starting around 1860 AD, resulting in an increase in P-loading in the lake and corresponding increase in primary production as measured by elevated organic matter in the lake sediment. Our results clearly show that lake eutrophication was concomitant with changes in the genetic structure of a Daphnia pulicaria population that was present throughout the entire

Figure 3 (a–c) Phosphorus (P)-retention efficiency (a), growth rate (b) and PUE (c) of nine resurrected Daphnia pulicaria genotypes. Individuals were resurrected from four sediment layers corresponding to different P-environments across recent and historical time periods. Three genotypes were tested from the 4–8 cm depth (2008–2002 AD); three genotypes from the 20–24 cm depth (1977–1967 AD); one genotype from the 52–56 cm depth (1646–1536 AD); and two genotypes from the 60–64 cm depth (1418–1301 AD). Standard deviations (SD) were calculated across individual clones and their replicates for each time period. (a) Mean ± SD decays per minute of nine clones (three replicates each) indicating retention of 32P by genotypes. (b) Mean ± ISD growth of nine clones (10 replicates each). (c) Mean ± ISD PUE of nine clones (5 replicates each).
Our observations on c. 700-year-old genotypes revealed no differences in P retention based on P supply. Although the small number of older genotypes available due to low hatching success precluded robust analyses and inferences regarding phenotypic evolution, the possibility for such striking phenotypic differences in P retention phenotype and its plasticity in response to P supply to be driven by evolutionary change cannot be ruled out.

South Center Lake (SC) has experienced other human-induced impacts (other than elevated P levels) that warrant discussion. SC has served as an important recreational lake dating back to the 1880s (Minnesota Pollution Control Agency 2012), especially for sport fishing. Stocking histories of various fish species have been well-documented, and fish community manipulations have been recorded since the mid-1920s. In *Daphnia*, P supply is known to interact with other selective pressures such as the type and magnitude of predation to determine fitness of genotypes (Jeyasingh & Weider 2005). Direct effects of human activity (e.g. fish stocking), or shifts in other key environmental parameters, in addition to P supply, associated with eutrophication (e.g. turbidity, and vulnerability to predators) can alter selection on growth and life history traits and could therefore underlie the striking differences in phenotypes reported here. The role of such environmental shifts in driving the remarkable genetic and phenotypic shifts observed here remains to be deciphered.

**CONCLUSIONS**

While this study provides an unprecedented long-term (i.e. centuries to millennia) view of human-induced impacts on microevolutionary responses in a natural population, we recognise there are several outstanding issues. Specifically, these observations are on a single lake and were limited by the number of ‘ancient’ genotypes that could be successfully hatched, established, and used for experimentation. Nevertheless, results from genetic analyses (as outlined above and in the supplementary material) clearly indicate a single population over our observed time-scale, with marked genetic divergence concomitant with a period of rapid cultural eutrophication of the lake’s catchment area. Phenotypic measurements, despite being from a limited number of resurrected genotypes over time, still revealed major shifts in ingested P retention in *D. pulicaria*, at a scale that has not been observed in any previous study of extant daphnids. Together, these observations strongly indicate evolutionary shifts in this population of *Daphnia*.

The replication of such a centuries-long time-scale in a multi-lake system is not trivial, given idiosyncratic differences among lakes in the abundance, preservation, and hatching success of diapausing eggs. Nevertheless, similar long-term studies, although challenging, will be critical in corroborating the results we have presented here. Direct observation of genetic and phenotypic dynamics of populations before, and in response to, anthropogenic environmental change, including the detection of the genetic basis of the stark phenotypic divergence between ancestors and descendants using new sequenc-


**SUPPORTING INFORMATION**

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