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Zweerus, Naomi L ; Sommer, Stefan ; Fontaneto, Diego ; Ozgul, Arpat

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Life-history responses to environmental change revealed by resurrected rotifers from a historically polluted lake

Naomi L. Zweerus · Stefan Sommer · Diego Fontaneto · Arpat Ozgul

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Abstract Life-history adaptations to environmental change can be studied retrospectively in organisms that produce dormant propagules using methods of resurrection ecology. Here, we investigated such responses in a planktonic freshwater rotifer, *Brachionus calyciflorus*. We resurrected 14 clonal lineages from resting eggs extracted from three distinct sediment layers—representing periods of high, medium and low copper pollution—of a previously contaminated lake (Lake Orta, Italy). We exposed the resurrected clones to four copper concentrations over 14 days and recorded population densities at 48 h intervals. If the original populations in Lake Orta had adapted to the changing pollution levels, we expected to find demographic evidence of this adaptation in the resurrected lineages. However, we found high clonal variation in population-growth dynamics, which was more pronounced within than between pollution

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Keywords *Brachionus* · Resurrection ecology · Adaptation · Population dynamics · Copper pollution

Introduction

Populations in increasingly polluted environments can respond in two mutually non-exclusive ways to survive in situ; they may either plastically adjust the phenotype to enhance tolerance or adapt genetically to acquire resistance to the pollutants (Lopes et al., 2004). While the former response may happen fast within a single generation, the latter only occurs across generations. Yet, resistance to pollutants can evolve over only a few generations (Medina et al., 2007), but may also be lost quickly through invasion of non-resistant genotypes once the environment has recovered (Hairston et al., 1999). Hence, species with short generation times—relative to environmental

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N. L. Zweerus (✉) · S. Sommer · A. Ozgul
Department of Evolutionary Biology and Environmental Studies, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland
e-mail: naomi.zweerus@gmail.com

D. Fontaneto
National Research Council, Institute of Ecosystem Study, Largo Tonolli 50, 28922 Verbania Pallanza, Italy

change—might actually be able to genetically track changes in the environment (Geerts et al., 2015).

Studying micro-evolutionary responses in wild populations is challenging as it requires the monitoring of populations in an often noisy environment over extended periods of time (e.g. Reznick et al., 1997; Hendry & Kinnison, 1999; Ozgul et al., 2010; Schoener, 2011). It is possible to shortcut such long-term study periods by investigating species with a dormant life stage, provided that individuals in these stages can be collected in the wild (e.g. in soils and sediments) and subsequently be resurrected in the laboratory, a research field termed *resurrection ecology* (Kerfoot et al., 1999). Lake sediments are particularly suited for resurrection methods as they allow reconstructing eco-evolutionary dynamics by linking sediment chronology to known environmental changes (Orsini et al., 2013). Notable in this context are the rotifer and cladoceran resting stages, which may remain viable in sediments for decades (e.g. Hairston, 1996; Hairston et al., 1999; Decaestecker et al., 2007; Piscia et al., 2012).

Resting eggs of monogonont rotifers allow populations to survive periods of adverse conditions and are produced by mictic (i.e. sexual) females, in case these females are inseminated (Wallace, 2002; Carmona et al., 2009). The resting eggs are then shed by the mothers and undergo a period of dormancy before they hatch into female juveniles. If mictic females are not inseminated, they produce haploid eggs that develop into males. The predominant mode of reproduction, however, is the parthenogenetic cycle during which amictic (i.e. asexual) females produce diploid eggs that hatch directly into female juveniles (for further details on the rotifer life cycle, see Wallace, 2002). This cyclical parthenogenetic life cycle, where parthenogenetic reproduction is ameiotic and results in clones in absence of mutation, indicates a highly adaptive potential to large temporal variation (King & Serra, 1998).

Lake Orta in Northern Italy has a well-documented history of industrial pollution and recovery and, as such, has become a model ecosystem for studies of effects of environmental change on aquatic organisms (Bonacina, 2001c; Bonacina & Baudo, 2001). The pollution started in 1926, when a rayon factory began to discharge copper- and ammonium-contaminated wastewater into the lake, which had immediate effects on the plankton and the fish communities (Calderoni et al., 1992; Bonacina, 2001a). From the 1950s onward, newly built

plating factories contaminated the lake further by disposing additional heavy metals such as chromium, aluminium, nickel and zinc (Baudo & Beltrami, 2001; Bonacina & Pasteris, 2001). While in-lake Cu concentrations rose steadily to a peak value of $108 \mu\text{g l}^{-1}$ in 1958, they gradually declined after the commissioning of a wastewater treatment plant to about $50 \mu\text{g l}^{-1}$ by the late 1960s (Bonacina, 2001a; Calderoni & Tartari, 2001; Piscia et al., 2012). The Cu concentration remained fairly stable throughout the 1970s, but began to decline slowly again after a new and more efficient treatment plant came into operation in 1981; by the late 1980s, the Cu concentration was down to just below $40 \mu\text{g l}^{-1}$ (Calderoni et al., 1992). Restorative liming, performed between May 1989 and June 1990, accelerated the process of recovery and eventually cleared the lake water from the heavy metals (Calderoni et al., 1992; Bonacina, 2001a, 2001b).

Resting eggs of the planktonic rotifer *Brachionus calyciflorus* Pallas can be found at all sediment depths in Lake Orta, ranging from the top layers down to the ones deposited during pre-industrial times (Piscia et al., 2016). Such a ubiquitous distribution of resting eggs indicates that *B. calyciflorus* must have been able to sustain viable populations at Cu concentrations that were much higher than the tolerance thresholds known from acute toxicity tests (reviewed in Snell & Janssen, 1995). However, while the latter tests are usually done in synthetic freshwater without feeding test rotifers, other organisms in the lake, such as algae, bind free copper and thereby reduce the concentration in the water column. Nevertheless, the persistence of *B. calyciflorus* in Lake Orta through time, in addition to the fact that only one mitochondrial haplotype was found before, during and after the pollution (Piscia et al., 2012), suggests that the Lake Orta population may have acquired increased tolerance to copper through natural selection.

In the present study, we investigated the life-history responses of *B. calyciflorus* clones resurrected from Lake Orta sediments. We cultured clonal lineages from three distinct sediment layers, reflecting periods of high (corresponding to 1960–1975), medium (corresponding to 1980–2000) and low copper pollution (from the year 2000 onward), and we subjected them to different levels of copper contamination. If the original rotifer populations in Lake Orta had adapted to the pollution levels experienced during the period in which the sediments were deposited, we expected to

find signs of that adaptation in the resurrected experimental populations. That is, we expected clonal lineages from the high-pollution period to be the most tolerant and clonal lineages from the low-pollution period to be the least tolerant to copper stress.

Materials and methods

Resurrection of clonal rotifer lineages

First, we established clonal lineages of *B. calyciflorus* following the resting-egg extraction and incubation protocols described in Sommer et al. (2016). We extracted eggs from four different sediment cores that were collected in 2012 in the Buccione (core 12/6), the Pettenasco (cores 12/10 and 12/11) and the Omegna (core 12/12) basins of Lake Orta (Baudo & Beltrami, 2001). Dating of sediment layers was performed by matching the differently coloured layers of each core to the banded pattern of a reference core collected in 2007. This reference core was originally dated using radioisotope analyses (for details, see Piscia et al., 2012). The imprecision imposed by our indirect dating method should not have invalidated the age estimate assigned to each rotifer lineage, since we used rather broad time spans for each age class (cf. pollution periods below). We cultured the clonal rotifer lineages in 50 ml plastic tubes (Sarstedt AG & Co., Nümbrecht, Germany) filled with soft, artificial freshwater and supplemented with green algae, *Chlorella vulgaris* Beijerinck (strain 211-11b, SAG culture collection of algae, Göttingen, Germany), as food source (for the chemical composition of the artificial freshwater and the culturing methods of algae, see Sommer et al., 2016). We kept all rotifer cultures in an incubator (Panasonic SGMIR-154) at 20 °C in darkness. Once a given clonal lineage started to reproduce sexually, we collected up to 100 resting eggs, which we stored at 4 °C in darkness in order to suppress hatching. From this egg bank, we used one resting-egg per clonal lineage to start the experimental cultures. By using such an egg bank, we were able to start all experimental lineages at the same time for each run.

Experimental procedures

We used a 3-by-4 factorial design with three levels of sediment age—hereafter referred to as the high-

pollution (years 1960–1975), the medium-pollution (1980–2000) and the low-pollution period (>2000)—and four copper treatments consisting of 0, 15, 30 and 45 $\mu\text{g Cu l}^{-1}$. The high-pollution period corresponds to the first phase of recovery from copper contamination (in-lake Cu concentrations went down from about 100 to 50 $\mu\text{g l}^{-1}$); the medium-pollution period corresponds to the second phase of recovery from copper contamination (from 48 to 3 $\mu\text{g Cu l}^{-1}$) and the low-pollution period corresponds to almost copper-free conditions ($\leq 2 \mu\text{g Cu l}^{-1}$). We subjected five clonal lineages from the high-pollution and the medium-pollution period and four clonal lineages from the low-pollution period to each of the four copper treatments, resulting in 56 experimental rotifer cultures. For logistic reasons (resting-egg availability and workload), we had to split the execution of the experiment into four runs, each including 3–4 clonal lineages. Lineages were tested as they became available and each lineage was tested at all Cu concentrations in a single run. These runs consisted of a 14 day pre-experimental period, followed by a 20 day experimental period; the latter was divided into a pre-treatment (6 days) and a copper-treatment phase (14 days). The food was initially set to 2×10^6 algal cells ml^{-1} and was renewed every second day (see below). Except for the addition of copper, culturing conditions were the same as described above.

At the end of the pre-experimental phase, we randomly selected 40 amictic, egg-carrying females from each clonal culture and transferred them in batches of ten individuals to new 50 ml tubes, one for each of the four copper treatments. This step marked the beginning of the experiment (day 0). On day 6, we started the different treatments by adding the corresponding amount of copper sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) to the cultures. Copper sulphate dissolves in water into bioavailable cupric ions and sulphate. Cupric ions and other labile copper species are considered to be toxic to zooplankton (Havens, 1994; Karlen et al., 2002). Since we did not measure cupric ion concentrations in the experimental tubes, we do not know the true concentrations of toxic copper species in the tubes. However, we assume that the toxicity of the culture medium increased with the amount of copper sulphate added (cf. Pradeep et al., 2015).

We monitored population densities during the entire 20 day experimental period by harvesting two

1 ml samples per culture every second day, starting on day 2. Prior to sampling, we inverted each tube to homogenise the cultures. We removed dead individuals from the samples, returned the resting eggs to the experimental cultures and preserved the samples by adding 1 ml of 100% ethanol for later counting of rotifers (see below). We sealed the samples with Parafilm® M to prevent evaporation and stored them at $\sim 14^\circ\text{C}$ in darkness. We replaced the harvested volume by adding 2 ml of a highly concentrated (50×10^6 algal cells ml^{-1}) food suspension, which was supplemented with the corresponding Cu concentration. In doing so, we provided fresh food at an effective density of 2×10^6 algal cells ml^{-1} every second day. We did not refresh the entire culture volumes due to the risk of losing individuals during the transfer and because of time constraints (in the case of transferring rotifers one by one) or the risk of cross-contamination (in the case of sieving).

From each harvested sample, we recorded the total number of female rotifers and, among egg-carrying females, distinguished between mictic and amictic individuals. We did not count the males, but we recorded whether they were present in a given sample. At the end of the experiment, we counted the resting eggs in all 50 ml tubes as an estimate of the investment into sexual reproduction of each culture.

Data analyses

For a qualitative assessment of population dynamics, we first plotted the density time series of all experimental populations. Visual inspection of these growth trajectories indicated that run number—the splitting of the experiment into four sequential runs—had no effect on the outcome; run number was therefore not included in further analyses.

For the quantitative comparison of population performances, we analysed the intrinsic (i.e. maximum per capita) population growth rate r . In order to calculate r for each experimental population, we first log-transformed the density data and then fitted a linear model to increasingly longer growth trajectories. We started with a time window spanning from day i (days after the beginning of the copper treatment) to day $i + 3$ and gradually increased the window by one time step. While creating the time windows, we limited i to <4 days to avoid missing an initial increase followed by a rapid decline. We then used the

coefficient of determination (R^2) to identify the period of exponential growth—the time window with the highest R^2 for the linear fit—and calculated r (i.e. the slope of the linear regression) within these time windows for each experimental population. Subsequently, we fitted linear mixed-effects models to estimate the effects of pollution period, copper treatment and their two-way interaction on r . Clone identity (1lclone) and interaction between copper treatment and clone identity (Cu1Clone) were included as crossed random effects. The pollution period and copper treatment were modelled as fixed effects. We tested all possible parameter combinations and used Akaike's information criterion (AIC) to identify the best approximating model. Finally, we performed a post hoc power analysis (Green & MacLeod, 2016) to determine the statistical power of rejecting the null hypothesis.

To assess the effects of pollution period and copper treatment on the investment into sexual reproduction, we analysed the numbers of resting eggs produced over the entire (20 day) experimental period. We used the same mixed-effects modelling approach as described above. In addition, to investigate the effects of population density and mictic ratio on resting-egg production, we calculated Spearman's rank correlation coefficient (r_s) between the number of resting eggs and the integrated number of females on the one hand and the mictic ratio on the other hand. The mictic ratio was calculated as the integrated ratio of mictic females to the population density through time.

Data analyses were performed using the software R, version 3.1.3 (R Core Team, 2015). The packages *lme4* (version 1.1-7; Bates et al., 2014) and *nlme* (version 3.1-120; Pinheiro et al., 2015) were used to fit the models.

Results

Inter-clonal variation was generally high, causing large differences in population-growth dynamics within pollution periods (Fig. 1). Clonal lineages from the low-pollution period showed a distinct bimodal distribution of population dynamics, resulting from differences between clones rather than between treatments. While two lineages from this period grew fast regardless of the Cu concentration, the other lineages grew consistently slowly. The differences in growth

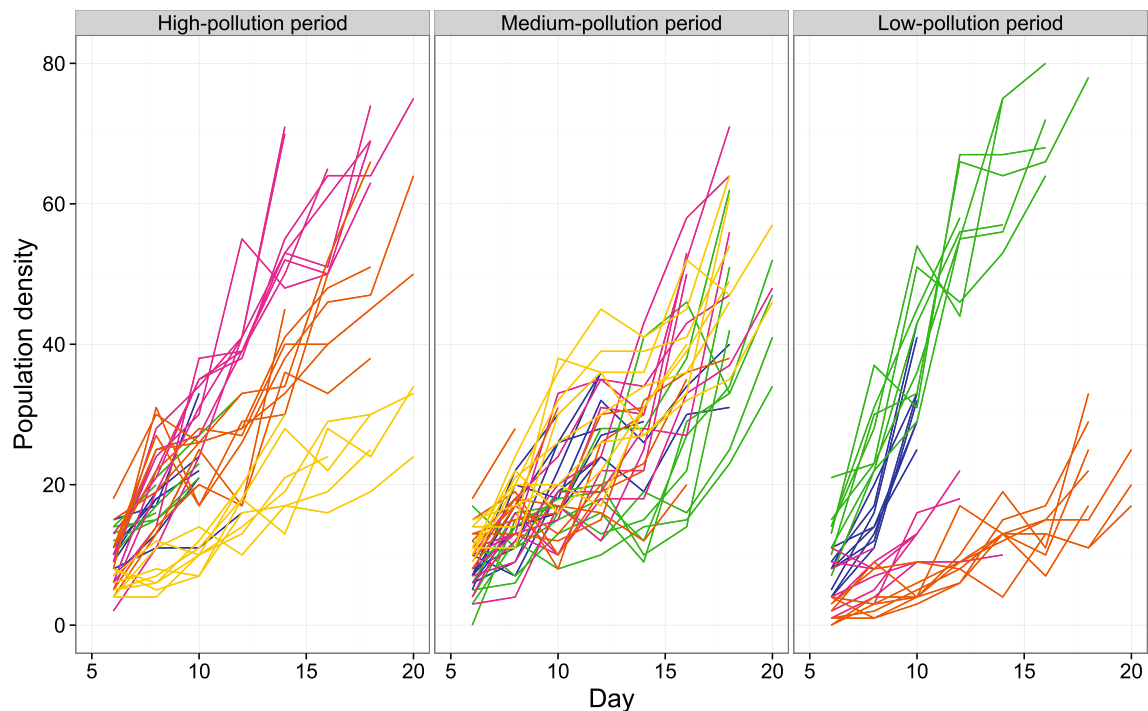


Fig. 1 Population densities (number of female rotifers per ml) from the start of the copper treatment (day 6) to the day each population reached the highest density within the 20 day study

dynamics of clonal lineages from the high-pollution period were less pronounced, mostly because one lineage grew at an intermediate rate. The difference between fast and slow growing clonal lineages was less pronounced in the medium-pollution period, resulting in a rather continuous distribution of overlapping growth dynamics.

The AIC-selected best model indicated that r was affected by the pollution period (model #3 in Table 1), yet we found no significant interaction between the pollution period and Cu (cf. model #5 in Table 1). All other models had comparatively little support ($\Delta\text{AIC} > 2$). Given our study design, the power to reject the null hypothesis, no layer effect, was 89.6% (CI 87.5–91.4). Moreover, median r -values tended to increase from the high- and medium- to the low-pollution period (Fig. 2). They were similar under the four copper treatments within the high-pollution period, but tended to be lower than in the respective control when lineages of the two other periods were exposed to copper (Fig. 2). The variation in r , on the other hand, was higher between clonal lineages from the high-pollution period.

period. Different colours represent individual clones ($N_{\text{high}} = 5$; $N_{\text{medium}} = 5$; $N_{\text{low}} = 4$). Each clone is represented by eight trajectories (four copper treatments, two subsamples each)

Table 1 Comparison of linear mixed-effects models testing the effects of copper treatment (Cu) and pollution period (period) on the intrinsic population growth rate (r) in resurrected rotifers

Model	Parameters	<i>df</i>	ΔAIC
#1	Constant	13	4.9
#2	Cu	16	7.8
#3	Period	15	0.0
#4	Cu + period	18	2.2
#5	Cu + period + Cu:period	24	11.9

Clone (1clone) and the Cu–clone interaction (Culclone) were included as crossed random effects in all models

df degrees of freedom, ΔAIC difference of a given model to the selected best model (#3, in bold)

Resting-egg production

As a measure for the investment into sexual reproduction, the numbers of resting eggs produced per clonal lineage in each treatment were counted immediately after termination of the experiments. The lineages from

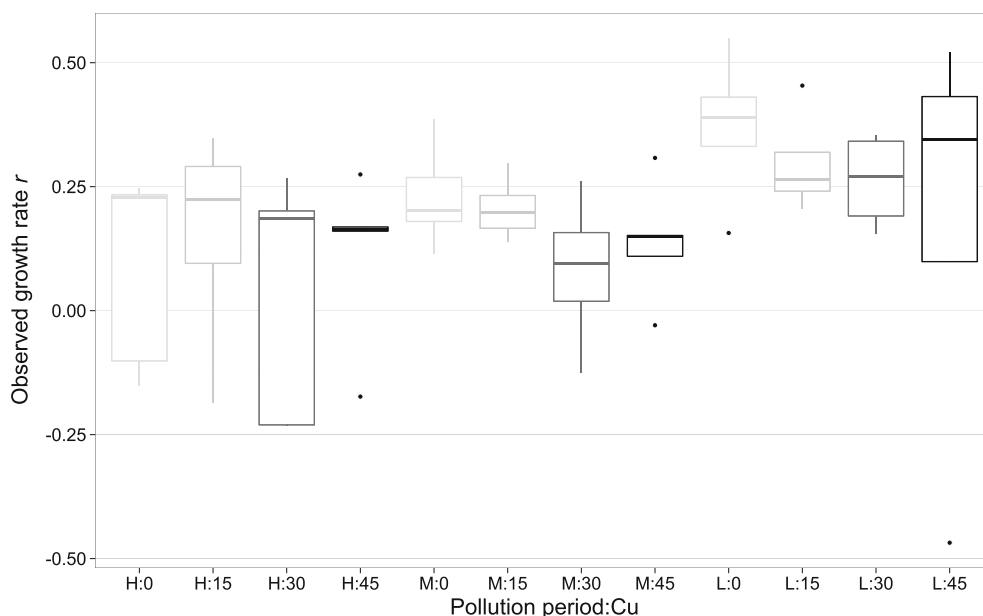


Fig. 2 Intrinsic population growth rates r (day^{-1}) for clonal rotifer lineages from three pollution periods (H: high pollution, M: medium pollution, L: low pollution) tested under four copper treatments (0, 15, 30 and 45 $\mu\text{g Cu l}^{-1}$). The lower and upper borders of the boxes represent the first and the third quartiles.

the high-pollution period produced the highest number of resting eggs, followed by lineages from the low-pollution period, whereas those from the medium-pollution period produced the lowest numbers of resting eggs (Fig. 3). Furthermore, in the latter pollution period, resting-egg number tended to decrease with increasing Cu concentration, while no clear pattern could be observed for the two other periods (Fig. 3). The selected best model included only the effect of pollution period (model #3 in Table 2). However, model #4 (Cu + period) was also substantially supported by the data ($\Delta\text{AIC} = 1.8$). Finally, we found a negative correlation between resting-egg number and total number of females per culture counted over the course of the experiment ($r_s = -0.60$, $P < 0.001$, $n = 56$; Fig. 4), but there was no significant correlation between resting-egg number and mictic ratio ($r_s = 0.11$, $P = 0.403$, $n = 56$).

Discussion

Combining the pollution history of Lake Orta with methods of resurrection ecology allowed us to explore the population dynamic responses of *B. calyciflorus*

The median values are indicated as lines within the boxes. Whiskers above and below the boxes extend to maximally 1.5 times the interquartile range; outliers are marked as dots ($N = 5$ per boxplot of the high- and the medium-pollution period; $N = 4$ per boxplot of the low-pollution period)

under controlled laboratory conditions. Under the adaptive evolution hypothesis, we expected clonal lineages from the high-pollution period to perform best under copper stress and clones from the low-pollution period to perform worst. However, although individual population-growth dynamics varied considerably, the variation was larger among lineages within pollution periods than within lineages among copper treatments (cf. clustering of same-colour lines in Fig. 1). Moreover, intrinsic population growth rates increased chronologically from the high- to the medium- to the low-pollution period. These results contradict the pattern we predicted, that is, they do not indicate any sign of adaptation to copper stress in the Lake Orta rotifers. As such, our findings are comparable to a study on *Daphnia* from Lake Greifensee (Switzerland), in which the authors found no evidence for adaptive change to a decrease in phosphorous concentration during oligotrophication of the lake (Spaak & Keller, 2004).

The lack of evidence of adaptive evolution might partly be explained by a phenomenon termed *genetic slippage*, which describes a shift in the genetic mean of a population in the direction opposing selection (Lynch & Deng, 1994). Such a shift is induced by the breakup of favourable gene combinations and might

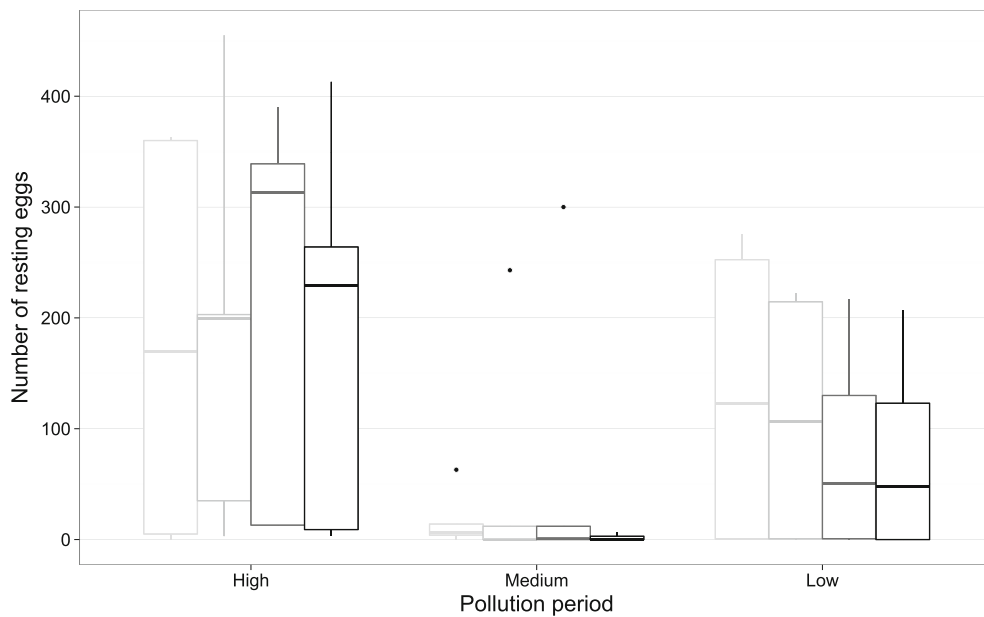


Fig. 3 Numbers of resting eggs produced by rotifers from three pollution periods cultured at four copper concentrations. Other conventions are as in Fig. 2

Table 2 Comparison of linear mixed-effects models testing the effects of copper treatment (Cu) and pollution period (period) on the resting-egg number, a measure of investment into sexual reproduction

Model	Parameters	df	Δ AIC
#1	Constant	6	24.9
#2	Cu	7	26.8
#3	Period	8	0.0
#4	Cu + period	9	1.8
#5	Cu + period + Cu:period	11	4.9

The model structure and conventions are the same as in Table 1

Model #3 (in bold) is the AIC-selected best model

occur whenever resting stages are produced through sexual reproduction. For example, it has been demonstrated in *Daphnia pulex* that the phenotypic shift in the onset of reproduction was actually in the same direction as the (presumed) selection (Lynch & Deng, 1994). Moreover, a number of other resurrection studies on *Daphnia* (reviewed in De Meester et al., 2007) successfully demonstrated adaptive evolution despite potential genetic slippage. We therefore believe that potential genetic slippage does not invalidate our conclusion that Lake Orta rotifers from different pollution periods did not differentially adapt to the respective copper levels.

We observed high clonal variation in population growth across all pollution periods. Because maternal effects were likely negligible after the 14 day (roughly three generations; cf. Halbach, 1970) pre-experimental period, this variation suggests that *B. calyciflorus* must have been able to maintain a considerable level of genetic variation, even at times when the lake was highly contaminated. This conclusion contrasts results from a resurrection study on *Daphnia* from Lake Constance (Hairston et al., 1999), in which the authors found reduced genetic diversity during periods of increased environmental stress (high prevalence of toxic cyanobacteria); clones with a low resistance were eliminated during that period. In Lake Orta rotifers, on the other hand, evidence for high genetic diversity during periods of high copper pollution comes from an increase in the number of resting eggs—including many hatched ones—embedded in the corresponding sediment layers (Piscia et al., 2016). The investment into sexual reproduction at this point of time might have allowed the population to increase the genetic variance by recombination (Pourriot & Snell, 1983; Lynch & Deng, 1994). Furthermore, high clonal variation suggests a bet-hedging strategy, which is considered to be common in diapausing organisms such as monogonont rotifers (Brendonck & De Meester, 2003; García-Roger et al., 2014). Since bet-hedging creates heterogeneity in

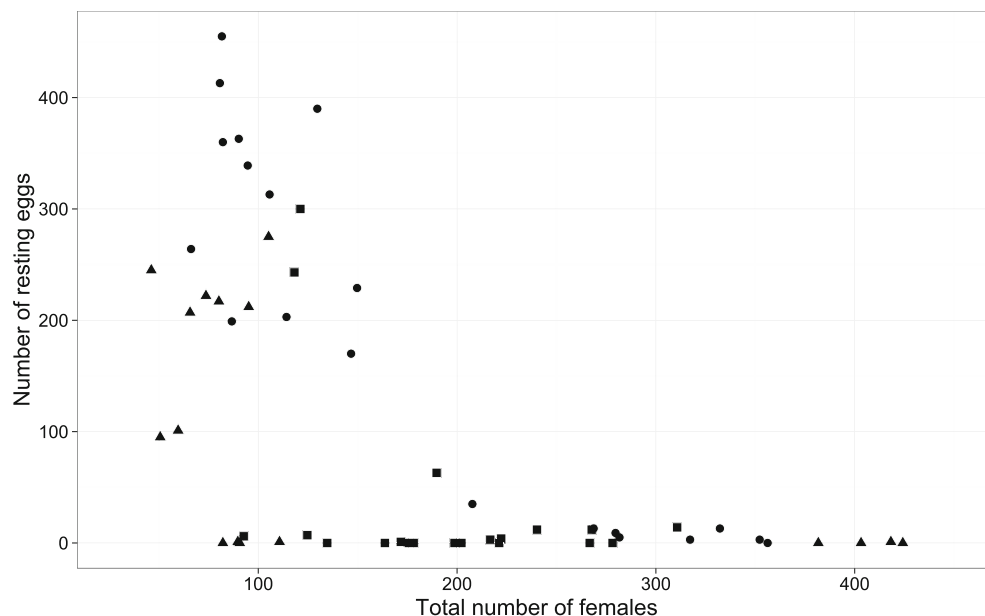


Fig. 4 Relationship between the total numbers of resting eggs produced till the end of the experiment and the total number of sampled females per experimental culture. Symbols indicate the pollution period of origin (circles high, squares medium, triangles low)

genotypes over time (Gilbert & Schröder, 2007), creating a genetically diverse reservoir of resting eggs at each generation is a successful strategy of rotifer populations in unpredictable environments. Such a strategy is consistent with the high heterogeneity found here, which was more pronounced within than between pollution periods.

Since we investigated clonal lineages after one round of self-fertilisation, inbreeding depression might have affected the performance of the experimental populations. Inbreeding depression caused by selfing is known from studies on both rotifers (Hertel, 1942; Birky, 1967; Tortajada, 2009) and *Daphnia* (Innes, 1989; De Meester, 1993; Lynch & Deng, 1994). In rotifers, inbred populations showed reduced viability, reduced hatching success and slower population growth. Similarly, inbred *Daphnia* showed reduced body size, clutch size and survival as well as greater variation in the latter parameter. Likewise, we observed heterogeneity in life-history traits such as hatching success, growth and maturation in the Lake Orta rotifers. Hence, the variation we found between clonal lineages might also partly be a result of inbreeding depression.

We expected clonal lineages from the most recent, low-pollution period (post-2000) to perform worse than older clones, because the original populations from this period were exposed to the lowest Cu

concentrations. Yet, the data revealed that only growth rates of lineages from the medium-pollution period (1980–2000) tended to decrease with increasing copper pollution. Strikingly, lineages from this period invested less into sexual reproduction compared to the lineages from the low- and the high-pollution period. While rotifers of the medium-pollution period were exposed to intermediate Cu concentrations, they experienced the most acidic conditions in the history of Lake Orta (Piscia et al., 2016), which might have imposed an additional and potentially stronger selection pressure than copper stress. If so, the different dynamics observed in resurrected clonal lineages from this period probably reflect a cost of acidification tolerance, a hypothesis worth testing in future studies.

Given what is known about effects of copper on *B. calyciflorus* from ecotoxicological studies (e.g. Snell & Moffat, 1992; Janssen et al., 1993, 1994; Sommer et al., 2016), the high copper tolerance observed in our study was rather surprising. While copper has been demonstrated to negatively affect the intrinsic population growth rate r at concentrations as low as $5 \mu\text{g Cu l}^{-1}$ (Janssen et al., 1994), in our study, copper reduced r mostly at the two highest copper concentrations. Hence, if the high copper tolerance observed in our study was not an artefact of the experimental methods, in future experiments with Lake Orta rotifers

it might be worth testing the full range of historic Cu concentrations, which extends to more than twice the maximum concentration used here.

Finally, we found a negative correlation between the number of resting eggs produced over the experimental period and average population size, resulting from a trade-off between immediate population growth via asexual reproduction and investment into sexual reproduction (Reznick, 1985; Stelzer, 2005). Mictic ratio, however, did not correlate with resting-egg number. Together, these results suggest that smaller populations, which produced more resting eggs, did not have a higher proportion of mictic females. This, in turn, implies that the larger number of resting eggs found in these populations must have been produced by a relatively low number of mictic females and that the efficiency of sexual reproduction in small populations was higher. On the one hand, these findings might appear counterintuitive, since the encounter rates of males and females are expected to increase with increasing population density (Snell & Garman, 1986). On the other hand, per-capita food availability must have been higher in populations with lower densities, which could explain the larger reproductive output of individual rotifers in these populations.

Conclusions

In this study, we resurrected *B. calyciflorus* clonal lineages from different Lake Orta sediments and compared their tolerance to four levels of copper pollution. Overall, we found high clonal variation in population-growth dynamics, which was more pronounced within than between pollution periods. Moreover, clones historically exposed to high-pollution levels did not perform better under experimental copper stress than clones from intermediate or low copper pollution periods. Thus, we did not find any evidence for adaptive evolution. The pattern we observed was actually reversed to the one we had expected: intrinsic population growth rates of resurrected rotifer lineages apparently increased with decreasing copper-pollution levels in Lake Orta.

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