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Lifetime Response of Contemporary Versus Resurrected *Daphnia galeata* Sars (Crustacea, Cladocera) to Cu(II) Chronic Exposure

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Abstract Resurrecting legacy lineages of organisms from sediment cores of known geological age allows us to understand how environmental change can cause selection pressures that constrain the variation of populations over time. We quantified the lifetime tolerance and effects of Cu(II) exposure on *Daphnia galeata* in a polluted subalpine lake by comparing extant individuals with those resurrected from ephippia extracted from ca. 30-years-old sediments. Laboratory experiments were conducted using two Cu(II) concentrations, 40 and 10 µg L^{-1}, corresponding to the levels recorded in the lake, during chemical recovery, when *Daphnia* first re-appeared and succeeded. Contemporary *Daphnia* were unable to survive after the 10th day at either of the Cu(II) concentrations, and were unable to successfully reproduce. *Daphnia* cohorts from the past performed better in low Cu(II) concentrations than in copper-free, control conditions. The copper-adapted, tolerant *Daphnia* strains grew faster under non-toxic conditions, but were unable to survive new pollution events.

Keywords Resurrection ecology · Neo- and paleo-ecotoxicology · Life strategies · Copper ecotoxicity · *Daphnia galeata*

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Resurrecting legacy lineages of populations from sediments of a known geologic age (resurrection ecology; Kerfoot et al. 1999; Kerfoot and Weider 2004) offers a unique opportunity for quantifying changes in the diversity, taxa composition and life strategies of aquatic communities in response to natural and anthropogenic disturbances. The resurrection of ancestors for transgenerational tests represents a new experimental paleolimnological approach that has the potential to test paleolimnological inferences directly (Kerfoot and Weider 2004), and add an often lacking dynamic (Jeppesen et al. 2001; Orsini et al. 2013) and ecotoxicological (paleo-ecotoxicology: Herkovits 2001) dimensions. Comparing the performance of past populations versus contemporary ones, under past and present conditions, allows us to detect changes in sensitivity levels, and to identify mechanisms responsible for the ability of species/clones to compete and survive through time.

The ease of culturing and hatching ephippial eggs, along with the crucial role zooplankton play in the transfer of matter, energy and pollutants through the pelagic food web, makes *Daphnia* a suitable taxon for resurrection ecology applications. *Daphnia* is a model organism in ecotoxicology and environmental genomic studies that are aimed at understanding genome-environment interactions (Colbourne et al. 2011). Because of their fast population growth rate, as many as 30 successive *Daphnia* generations may live and die in a lake in a single year. A section of a sediment core dating back to half a century ago, allows us to compare the life history patterns of organisms from ca.

1500 generations ago, with respect to those presently living in the same environment. The span of viable eggs retrieved from the sediment cores fits in with the timeframe for microevolutionary response, offering indirect evidence for microevolutionary changes, along with environmental conditions.

Adverse effects of metal contamination include death of the organism due to direct toxic effects, replacement of sensitive species/strains by less sensitive ones, shifts in food-web interactions, acclimation of species/strains to stress and selection of genetically inherited tolerance (Belfiore and Anderson 2001; Bossuyt et al. 2005; Guan and Wang 2006; Klerks and Weis 1987). Previous studies (see details in Tsui and Wang 2007) have shown that *Daphnia magna* can develop a tolerance to metals by the induction of metallothionein-like proteins, which can sequester toxic metals and so reduce their uptake. However, Shaw et al. (2006) highlighted differences in tolerance development among different species of zooplankton exposed to the same metals. Research on the ecotoxicological responses of different zooplankton species can be useful to better understand the development of tolerance.

In this study, we quantified the tolerance and effects of lifetime exposure to [Cu(II)] of past versus contemporary *Daphnia galeata* lineages from Lake Orta, a deep, subalpine lake in Italy. We exposed past and contemporary *Daphnia* to two different concentrations that were detected during lake recovery from heavy, chronic copper pollution (Piscia et al. 2009). We aimed to determine whether tolerance to copper exposure was higher in past versus present organisms, as well as attempt to identify any changes in life strategies of *Daphnia* during the early and late lake recovery phases. We expected contemporary *Daphnia* populations to perform better in present-day, non-toxic conditions, and to exhibit less tolerance to copper exposure than past, resurrected *Daphnia*. As adaptation to copper exposure implies a cost, we also hypothesized that past *Daphnia* would perform better under copper free conditions than under copper exposure.

Materials and Methods

Lake Orta was notorious for its chronic pollution, caused by the discharge of huge amounts of $CuSO_4$ and $(NH_4)_2SO_4$ from a rayon factory (Calderoni et al. 1990) in 1926. In-lake nitrification processes in a poorly buffered environment, caused a strong acidification (to pH 4.0), along with increasing ionic copper concentration (to a maximum of 85 μgL^{-1} during winter mixing). Stopping the discharge of pollutants' in 1980, and a liming intervention in 1989–1990, accelerated chemical recovery, after which biotic re-colonization took place. The acid-tolerant species *D. obtusa* (Fryer 1985), not previously observed in the lake, appeared when the lake was acidic. Allozyme analyses revealed that the population consisted of a single clone (Bachiorri et al. 1991a, b; Bonacina et al. 1994; Rossi et al. 1993). After neutral pH conditions were re-established, *D. longispina* gr. of the same morphotype as before lake pollution was identified based on morphological traits (Benzie 2005; from 1996 to 2001; Bonacina 2001). However, in 2005 and 2011, genetic analyses identified the species to be *D. galeata* (Piscia et al. 2006; Wolinska et al. 2007), as they are sibling species, with a high phenotypic plasticity.

Ephippia were extracted from sediment core (ORTA 07/1A) sections deposited during the early recovery phase of Lake Orta from pollution, corresponding to ca. 30–40 μg/L [Cu(II)] (Manca and Comoli 1995; Calderoni and Tartari 2001), as the source of *D. galeata* from the past (Piscia et al. 2012). Contemporary and past females were individually grown in the laboratory at 20 ± 1°C, in a 16L:8D photoperiod in 0.45 μm filtered, aerated surface water from the lake with 22×10^3 cell/mL/day *Kirchneriella subcapitata* Korshikov (Manca and de Bernardi 1987; Bossuyt and Janssen 2003, 2005). From contemporary and past females, clonal lineages were obtained (C and P), and acclimated to laboratory conditions until the third generation (F3) to rule out maternal effects (de Bernardi and Manca 1981; Lampert 2001).

Experimental cohorts (10 individuals each) were established from ≤24 h old (F4) new-born contemporary (C) and resurrected (P for "Past") *Daphnia*. Two sub-lethal copper concentrations ($L = 10\ \mu g\ L^{-1}$ [Cu(II)] and $H = 40\ \mu g\ L^{-1}$ [Cu(II)]) were tested against controls (hereafter CC and PC, respectively; Ponti et al. 2010). We measured and randomly assigned individuals of each cohort to one of the treatments or the controls. We kept the single specimens in the culture medium (100 mL) renewed every other day, and we inspected them for numbers of survivors, eggs, neonates and female survival. The culture medium was the same as that used to rear the organisms in the laboratory, with the addition of $Cu(SO)_4$ stock solution ($Cu = 100\ mgL^{-1}$).

Total Cu concentrations were determined at times 0, 24 and 48 h (i.e., times during the period for each renewal of culture medium during life table experiments) by inductively coupled plasma–mass spectrometry according to EPA method 200.8 (ICP–MS, model X Series II from Thermo; see Monticelli et al. 2011 for experimental details). A protocol of QA/QC was strictly followed, including allowing a 1 h warm up time, mass calibration of the instrument, check of sensitivity and signal stability by tune solutions, blank measurements, calibration for each analysis batch, control charts, analysis of synthetic samples, as well as participation in inter-calibration exercises. The limit of detection for Cu was

0.02 μg/L determined according to the IUPAC recommendation (3 times the blank standard deviation divided by the sensitivity). Relative percentage standard deviations for Cu measurements in the samples were in the range 0.5 %–2 % (n = 5). Recovery of Cu from culture mediums amended with low (10 μg/L) and high (40 μg/L) copper concentrations were quantitative.

We recorded age at first reproduction, age at death, age-specific egg clutch size and sexual production (newborn/clutch dry weight over female lifespan). Weight was obtained by applying LWRE (Length Weight REgression, Manca and Comoli 2000) to newborn (<12 h from release). We tested the effect of different treatments on survival, fecundity and offspring body size of individuals in C and P cohorts using ANOVA. We used clutch order as a covariate in comparison with newborn body size. All statistical analysis were performed using SPSS 19.0 software (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.).

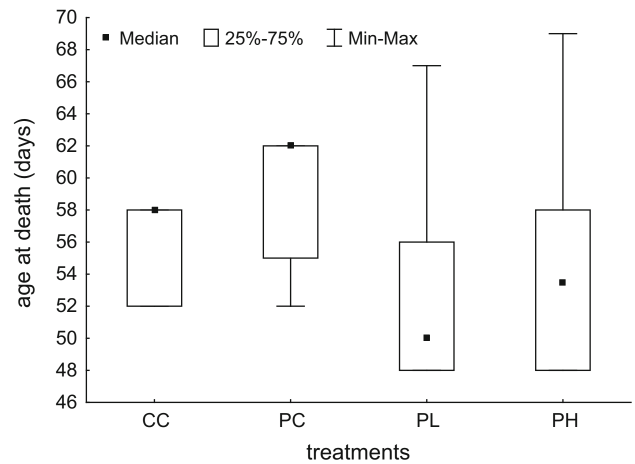

Fig. 1 Mean age at death of contemporary *Daphnia* in control (CC) and resurrected *Daphnia* in control (PC) experiments, exposed to 10 μg L^{-1} Cu (PL) and 40 μg L^{-1} Cu (PH). CL and CH treatments are not shown as all individuals died after 10 days of exposure

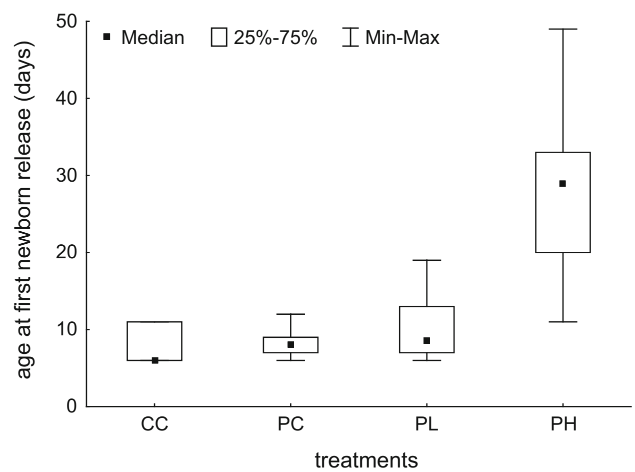

Fig. 2 Age at first release of new-born contemporary *Daphnia* in control (CC) and resurrected *Daphnia* in control (PC) experiments, exposed to 10 μg L^{-1} Cu (PL) and 40 μg L^{-1} Cu (PH). CL and CH treatments are not shown as none reproduced

Results and Discussion

Our results are referred to nominal [Cu(II)] concentrations, because no significant differences (ANOVA $p < 0.005$) were detected between nominal and analytical concentrations during the 48 h exposure, i.e. the time frame between two subsequent renewals of the culture medium. Differences in body size of C- and P-cohorts at the beginning of the experiment were not statistically significant ($F_{1,58} = 2.152$ $p = 0.148$). All contemporary *D. galeata* died after a 10-day exposure to L- and H- [Cu(II)] and none reproduced at the high copper concentration (CH). At the lower copper concentration (CL), two out of ten females released the first clutch of eggs, of two and three eggs per clutch, respectively, though these did not hatch. In the control sample, the age at death of contemporary *Daphnia* (CC) was 56.2 days (SD = ±2.90), the mean total offspring number per female was 79.3 (SD = ±21.43) and the age at first egg release was 7.6 days (SD = ±2.37). Abortion percentage per female was low, at between 0 % and 4 %.

Past, resurrected cohorts (of both, PL- and PH-females) survived, grew and reproduced. Between-treatment and control age at death did not significantly change (PC = 58.4 ± 0.15 days, PL = 53.3 ± 0.28 days, PH = 55.7 ± 0.11 days; $F_{2,27} = 1.85$ $p = 0.173$; Fig. 1). PH-females released their first offspring later (29.4 ± 1.52 days; Fig. 2) and in a lower number ($n_{newborn} = 15.10 \pm 1.55$) than PL- and PC-females (38.90 ± 2.78 and 33.20 ± 1.99, respectively; $F_{2,27} = 10.456$ $p < 0.001$; Fig. 3). Overall, the difference in fecundity was not statistically significant ($F_{2,27} = 3.758$ $p = 0.066$) among treatments (Fig. 3). Abortion percentages varied between 0 and 30 % and mean values were not significantly different among treatments ($F_{2,26} = 2.839$ $p = 0.077$).

Mean age at death and mean age at first new-born release did not differ between CC- and PC-females (for age at death $F_{1,18} = 2.897$ $p = 0.106$; for age at first new-born release $F_{1,18} = 0.535$ $p = 0.474$; Figs. 1 and 2). Fecundity was significantly higher in CC- than in PC-females (79.7 ± 2.4 and 33.20 ± 2, respectively; $F_{1,18} = 29.433$ $p < 0.001$; Fig. 3). Offspring mean size and dry weight were not related to clutch order (for size: $F_{1,272} = 0.048$ $p = 0.827$; for dry weight: $F_{1,272} = 0.159$ $p < 0.690$). Abortion percentage varied between 0 % and 30 % and mean values were not

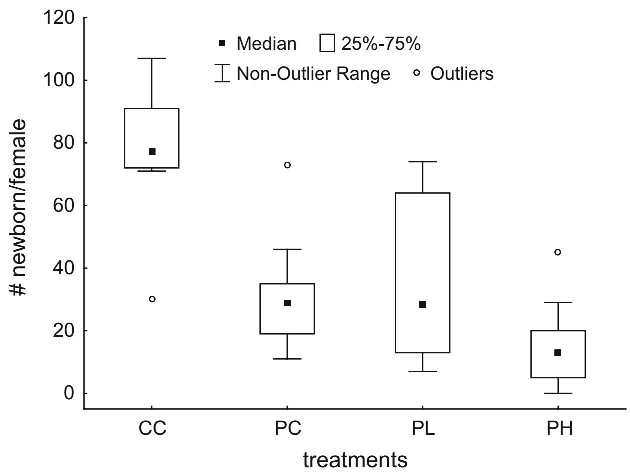

Fig. 3 Total number of new-born/female contemporary *Daphnia* in control (CC) and resurrected *Daphnia* in control (PC) experiments, exposed to 10 μg L^{-1} Cu (PL) and 40 μg L^{-1} Cu (PH)

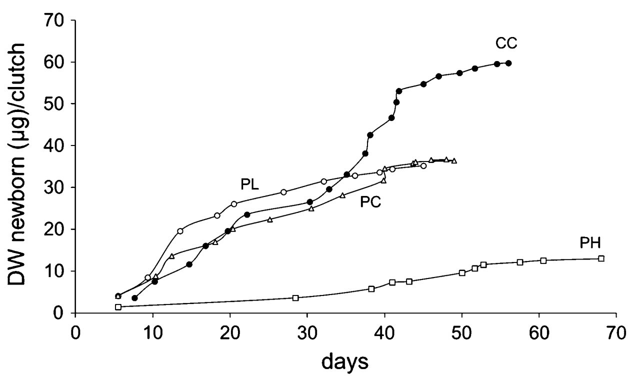

Fig. 4 Sexual production, expressed as dry weight (μg) of new-born release per clutch, of contemporary (CC) and past *Daphnia* (PC) in the control experiment, and of past cohorts exposed to 10 μg L^{-1} (PL) and 40 μg L^{-1} (PH) [Cu(II)]

significantly different between lineages ($F_{1,18} = 2.428$ $p = 0.137$). Sexual production was ca. double in CC (60 μg dry wt) than in PC (30 μg) and PL (37 μg; Fig. 4). The lowest sexual production was observed for past specimens in H-treatment (13 μg dry wt) as a consequence of delayed egg release and a lower per clutch production.

Combining paleo- and neo-ecotoxicological approaches help in understanding the response mechanisms of organisms exposed to pollution. CL and CH *Daphnia* cohorts were unable to tolerate chronic exposure. Contemporary-*Daphnia* survived both Cu(II) exposure concentrations for only 10 days, after attempting to reach sexual maturity. The few that did reach sexual maturity (CL) were unable to leave a progeny, as their eggs did not hatch. In addition, the release of eggs was delayed by 3 days with respect to the C- control and the P-*Daphnia* cohorts.

In previous experiments, contemporary Lake Orta *Daphnia* survived acute (48 h) toxicity exposure to 87 μg L^{-1} Cu(II) (Ponti et al. 2010), a concentration double the highest we tested in the present experiments. However, high tolerance to acute copper toxicity is confirmed by the present experiments, as all *Daphnia* cohorts were able to tolerate 10 days exposure to copper, even at the highest concentration tested (40 μg L^{-1}). The effect of chronic exposure became evident when *Daphnia* attempted reproduction. In addition, C-*Daphnia* cohorts were obtained from Lake Orta females sampled ca. 3 years later than those collected for acute toxicity tests by Ponti et al. (2010). This timeframe is compatible with microevolutionary processes, as ca. 90 generations could have occurred, justifying their loss of ability to survive under chronic high copper concentration exposure.

The two tested concentrations (10 and 40 μg L^{-1} Cu(II)) were representative of the pollution levels at the time *Daphnia* produced the ephippia resurrected as P-cohorts. The sediment section of core ORTA 07/1A used to extract ephippia dated back to 1986–1992, when in-lake copper concentration was between ca. 30 and 40 μg L^{-1} Cu(II) (Piscia et al. 2012). By choosing to establish cohorts from F4 females, we could rule out maternal effects (Arbačiauskas and Lampert 2003), which suggests that the best fitness of P- versus C-*Daphnia* cohorts under low copper concentration might result from a permanent resistance, induced by chronic exposure. In turn, the delayed and depressed fecundity of the PH cohort suggests that H-[Cu(II)] tested could be too high, and not representative of environmental conditions of resurrected *Daphnia*.

Accordingly, copper resistant P-*Daphnia*, were likely succeeded by non-resistant individuals once copper was not present in the lake. We found a significant trade-off between the costs involved in the process of surviving copper exposure and the number of progeny left by mothers during their life span (e.g. Atienzar et al. 2001). By investigating lifetime response, we were able to detect effects of Cu(II) exposure on the timing of egg release and number of *Daphnia* offspring. We hypothesized that toxicants at sub-lethal concentrations might act on reproductive strategies (Agra et al. 2010, 2011). The response that we observed was similar to that found under lowered or fluctuating temperature and food conditions (Manca et al. 1986). With increasing copper concentration, *Daphnia* tended to delay and decrease the number of offspring produced. Increased individual variability between P-cohorts (Figs. 1, 2, 3 and 4) also suggests a strategy for increasing the survival chance of a parthenogenetic population during pollution. This ability to cope with copper lifetime exposure realized at the expense of fecundity, might suggest a trade-off between copper tolerance and fecundity (e.g. Atienzar et al. 2001).

We expected to find a substantial enhancement in performance, particularly in sexual production, of P- resurrected females exposed to present, copper-free conditions. That we did not observe this, as the performance of PC cohort was substantially lower than that of PL-cohort, suggests that the mechanisms activated under pollution were not switched off under non-toxic conditions. The low copper concentrations at which P-cohorts perform best, likely represent the environmental conditions during which the ephippia were produced. According to our results, contemporary individuals would outcompete the P-resurrected *Daphnia* strain, when occurring together. By applying resurrection ecology, we were able to travel back in time to address *Daphnia* response patterns to copper pollution and recovery, to find out how contemporary organisms might react to new threats.

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