Ecotoxicology (2016) 25:708–719
DOI 10.1007/s10646-016-1629-7

Diapause as escape strategy to exposure to toxicants: response of *Brachionus calyciforus* to arsenic

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Accepted: 11 February 2016 / Published online: 20 February 2016

Abstract Invertebrate organisms commonly respond to environmental fluctuation by entering diapause. Production of diapause in monogonont rotifers involves a previous switch from asexual to partial sexual reproduction. Although zooplankton have been used in ecotoxicological assays, often their true vulnerability to toxicants is underestimated by not incorporating the sexual phase. We experimentally analyzed traits involved in sexual reproduction and diapause in the cyclically parthenogenetic freshwater rotifer, *Brachionus calyciflorus*, exposed to arsenic, a metalloid naturally found in high concentrations in desert zones, focusing on the effectiveness of diapause as an escape response in the face of an adverse condition. Addition of sublethal concentrations of arsenic modified the pattern of diapause observed in the rotifer: investment in diapause with arsenic addition peaked earlier and higher than in non-toxicant conditions, which suggests that sexual investment could be enhanced in highly stressed environmental conditions by increased responsiveness to stimulation. Nevertheless, eggs produced in large amount with arsenic, were mostly low quality, and healthy-looking eggs had lower hatching success, therefore it is unclear whether this pattern is optimum in an environment with arsenic, or if rather arsenic presence in water bodies disturbs the optimal allocation of offspring entering diapause. We observed high accumulation of arsenic in organisms exposed to constant concentration after several generations, which suggests that arsenic may be accumulated transgenerationally. The sexual phase in rotifers may be more sensitive to environmental conditions than the asexual one, therefore diapause attributes should be considered in ecotoxicological assessment because of its ecological and evolutionary implications on lakes biodiversity.

Keywords Hatching success · Heavy metals · Mixis ratio · Rotifers · Toxicity

Introduction

Many organisms live in remarkably unpredictable environmental conditions that restrict their growth, survival and reproduction. A very common response of some invertebrate organisms to environmental fluctuation is to enter into diapause, a reversible stage of reduced metabolic activity (Lennon and Jones 2011). The ecological and evolutionary relevance of diapause rests in that (1) this strategy promotes dispersal and colonization of new environments by passive transport of diapausing states, and (2) it permits overcoming harsh environmental conditions since diapausing states remain viable for decades, forming resting state banks (Marcus et al. 1994; Kotani et al. 2001; Cáceres and Tessier 2004; García-Roger et al. 2006). Banks of diapausing states contribute to prevent population extinction, supporting long-term species diversity, which could be a key aspect in allowing competitor coexistence (Chesson and Warner 1981; Cáceres 1997), while serving as a reservoir of genetic diversity. Also, incomplete termination of diapause has been considered a bet-hedging strategy of the individual producing dormant offspring, thus spreading risks (Venable 2007; García-Roger et al. 2014).

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Two important zooplankton groups—cladocerans and monogonont rotifers—are facultatively sexual. They typically have an exclusively asexual reproduction period followed by another in which sexual and asexual reproduction occur. In monogononts, asexual females, also called amictic females, produce asexual daughters by ameiotic parthenogenesis, thus causing clonal proliferation. Episodically, asexual females produce sexual daughters, also called mictic females. Sexual reproduction or 'mixis' has been proved to be density-dependent in several monogonont species, and appears to be induced by a chemical, a quorum-sensing molecule produced by the females themselves (Gilbert 1963, 2003; Snell and Boyer 1988; Carmona et al. 1993, 1994; Stelzer and Snell 2003, 2006; Fussmann et al. 2007). If a young sexual female is inseminated, her fertilized eggs develop to give rise to diploid diapausing embryos, called diapausing or resting eggs; otherwise the eggs develop to produce haploid males, which are dwarf and physiologically constrained.

Cladocerans of the genus *Daphnia,* and to lesser extent rotifers of the genus *Brachionus*, have been frequently used to detect effects by pollutants due to their sensitivity to various toxicants, their short lives and their important role in the ecosystem (Dahms et al. 2011). Nevertheless, because only the asexual portion of the life cycle is commonly investigated in toxicity studies, the true vulnerability of zooplankton to toxicants is often underestimated (Preston and Snell 2001; Navis et al. 2015). Several studies assessed the effects of pesticides, heavy metals, and endocrine-disrupting chemicals on sexual reproductive parameters of rotifers (Snell and Carmona 1995; Preston et al. 2000; Yoshinaga et al. 2000; Preston and Snell 2001; Radix et al. 2002; Marcial et al. 2005; Ríos-Arana et al. 2007) and cladocerans (Tatarazako and Oda 2007; Navis et al. 2013, 2015). Their results showed that, depending on the species and contaminants tested, reproductive parameters such as mixis ratio (i.e., proportion of sexual females relative to all females, sexual and asexual) and fertilization, and diapause parameters such as resting egg production and resting egg hatchability are among the most sensitive endpoints to assess toxicity in zooplankton species. Nevertheless, these results do not account for diapause as an evolutionary strategy to face hazardous conditions.

Within populations, individuals are indirectly affected by transgenerational transmission of the effects of contaminants (Massarin et al. 2010). Tolerance to different adverse environmental conditions depends upon the history of parental generations. On one hand, it was reported that offspring of populations exposed to pesticides evolve to higher tolerance (see Relyea and Hoverman 2006; Gustafsson et al. 2005). On the other hand, pollutants in the maternal environment of some aquatic invertebrates produce low quality offspring, which may be explained by high investment in processes of reparation and detoxification in the parental generation (Marshall 2008; Fernández-González et al. 2011). Therefore, it is expected that environmental conditions to which mothers have been exposed will affect the performance of their offspring, even if the condition has already disappeared.

Desert zones are commonly characterized by naturally high concentrations of minerals (Laurent et al. 2008). The region including Southern Peru and Northern Chile is one of the driest places in the world. High evaporation in the region makes continental water highly saline, with elevated concentrations of heavy metals such as lead, copper, cadmium and zinc, and metalloids such as arsenic (Pell et al. 2013). Additionally, mining operations are intense in the region, mainly focused on copper; these contribute to presence of the metals in the waters (Díaz-Palma et al. 2012). Not surprisingly, biodiversity in lakes near mining areas is greatly reduced, and zooplankton community structure shows strong particularities (Weisse et al. 2011, 2013). In past decades, mining and the concomitant arsenic concentration in the environment have been associated with severe damage to human health, especially in rural populations (Naujokas et al. 2013; Yañez et al. 2015). Arsenic is at the top of the Harzardous Substances Priority List published by Agency for Toxic Substances and Disease Registry (2013). Although not all arsenic chemical species are toxic, high toxicity of inorganic arsenicals has been shown in aquatic species of different trophic levels (Chen et al. 2000; Ríos-Arana et al. 2007). Data concerning to freshwater zooplankton communities in southern latitudes are often scarce, partial and biased by groups and geographic zones (Oyanedel et al. 2008). Nevertheless, knowledge of the ecology of the monogonont *Brachionus calyciflorus* in Chile and Argentina suggests higher abundance and frequency than other species of the plankton community (Kutikova and Fernando 1995; Acuña et al. 2008; Claps et al. 2011).

In this study we test whether sexual reproduction and production of diapause eggs is affected by the arsenic concentration in the monogonont rotifer, *Brachionus calyciflorus*. We take into account several consecutive generations, including those mediated by sexual reproduction (i.e., after diapause). Besides investigating the effects of arsenic on rotifer demographic, we aim to get insight into the possibility that an adverse condition may cause an escape response; i.e., diapause, and therefore represent an adaptive advantage in that condition. As sexual reproduction is density-dependent in ways that could differ among within-species strains, we first assessed the conspecific density required to induce the sexual reproduction in our experimental animals (Gilbert 1963, 2003; Snell and Boyer 1988; Carmona et al. 1993, 1994; Stelzer and Snell 2003, 2006; Fussmann et al. 2007).

Materials and methods

Cultures of the test species

Individuals of the herbivore rotifer *B. calyciflorus* were isolated from Tranque Lo Orozco, Chile (33°22′S–71°41′W), a permanent pond not exposed to mining activity. A clone was maintained under standard laboratory conditions (pH 7.5 ± 0.1, temperature 22 ± 1 °C, photoperiod 12:12 l:d) for 2 years prior to experimental use. For maintenance and experimental cultures we used COMBO medium (Kilham et al. 1998). Density was controlled in stock cultures, avoiding high densities before the experiments. Stock rotifer cultures were fed daily with the green microalga *Pseudokirchneriella subcapitata* at a density of 1×10^6 cell mL^{-1}, which was cultured with Bold's basal medium (Borowitzka and Borowitzka 1988). The microalga strain was obtained from the Ecotoxicological Research Center (CIE) of the Universidad Mayor, Chile, and maintained in isolation under standard laboratory conditions. The algae used as food were harvested during the exponential growth phase and their concentration was measured by direct counting.

Preliminary bioassays

Very high arsenic concentration (up to 3000 ng mL^{-1}) are found in some rivers of pre-Andes water in mining areas of northern Chile (Queirolo et al. 2000). The toxicant was incorporated into the treatments as As_2O_3 because this is a compound commonly found in aerosols produced by copper smelters that is dispersed by air, contributing to the contamination of water bodies (Pell et al. 2013). As^{3+}, the state in which arsenic is found in As_2O_3, is a very common inorganic species of arsenic found in the continental waters in the desert of northern Chile, it is the most mobile As state in the environment due to its high lability, and is highly toxic for most life forms (Díaz-Palma et al. 2012). Preliminary bioassays were conducted to define the experimental concentration of the As_2O_3 to use in the experiments.

A standard bioassay of population growth inhibition of the microalga *P. subcapitata* was conducted for 96 h, following the guidelines described by OECD (2011), and an acute toxicity bioassay was conducted with the rotifer *B. calyciflorus*, following ASTM (1991). From that data, 50 % reduction of population growth (EC50) and 50 % lethal concentration (LC50) were estimated for the microalga and rotifer, respectively.

Induction of sexual reproduction by crowding cues

Life table experiments were conducted to assess the effect of population density on the production of sexual females of *B. calyciflorus*. Two combined factors were studied: crowding cue level, which was established by rotifer density in the culture and food concentration. Five nominal crowding levels were tested by monitoring cultures started with single females confined in controlled volumes. Culture volumes were 50 mL, 200 µL, 100 µL, 50 µL and 25 µL, thus experimental densities were 0.002, 5, 10, 20 and 40 ind mL^{-1}. A density of 0.002 was considered as a control, assuming a negligible effect of this density on mixis. Food concentrations were 0.5×10^6 cells mL^{-1} (low) and 2.5×10^6 cells mL^{-1} (high). Ten females and their asexual descendants were used for each of the 10 (five crowing levels × two food concentrations) experimental combinations. Experimental cultures started with a neonate (<6-h old) female, which was checked daily. These individuals were our P generation. According to their first offspring, they were classified as sexual or asexual; the latter were monitored until death and their offspring were collected daily, being our F_1 generation. The procedure used for P, was repeated for F_1, F_2 and F_3, except that in F_3 monitoring ended after classifying the females as sexual or asexual. Therefore, mixis ratio (proportion of sexual females over all females) was measured over four successive generations. When we refer to the mixis ratio of a given generation we mean the proportion computed after classifying the females in that generation, i.e., the proportion produced by the previous generation. Statistical analysis was performed by factorial ANOVA and Student–Newman–Keuls (SNK) for multiple comparisons, repeated for the different generations. This experiment was used to estimate a population density that, even though relatively low, is able to induce sexual reproduction. To discount dependence within each lineage used in this experiment, we computed correlations between mixis ratios in F_2 and F_3 within-experimental combinations. These two generations were chosen because we obtained a high number of replicates with sexual reproduction. For this correlation, variates were mixis ratio in F_2 and average mixis ratio for the asexual daughters in F_3. Cases with no sexual females in both generations were discarded.

Effect of arsenic on diapause eggs production and quality in mass cultures

The experiment was conducted with two factors: arsenic addition (with/without) and food level (low and high, as above), with five replicates per experimental combination. Arsenic was added at 20 µg L^{-1} and the actual concentration of As^{3+} in the medium was estimated using HG-AAS (Anthemidis et al. 2005). Cultures were initiated at a density of 0.4 ind mL^{-1} to allow the exponential growth of the experimental populations. Each culture was initiated with 100 rotifer neonates in 250 mL of medium previously conditioned with crowding cue, prepared as follows.

Conditioned medium with crowding cue was prepared every day by culturing *B. calyciflorus* at a fixed density. The rotifer population density used to prepare the crowding cue was established from the results obtained from the experiment described above. After 24 h individuals were filtered out and the medium was membrane-filtered (0.45 μm), pH adjusted to 7.5 ± 0.1 and used as medium in the experiments. Conditioned medium was used to expose the cultures to mixis-inducing signal in all treatments from the beginning of the experiments. Rotifer density in the experimental cultures was assessed daily, either by exhaustive counting or by counting three to four 10 mL samples. Additionally, diapausing eggs were counted and removed from the container daily. Rotifers were transferred to new jars containing fresh medium with the corresponding experimental condition. Diapausing eggs were stored at 4 °C in darkness. Cultures were finished at day 18th, when constant or declining density was commonly observed. Instantaneous growth rate *r* (per day) was estimated as the slope of the curve $ln\ N_t$ versus time in the exponential growth phase. The exponential growth phase was determined based on the highest determination coefficient of $ln\ N_t$ *vs* time. Effects on *r* were tested using two-way ANOVA, after verifying parametric assumptions, and the Holm-Sidak post hoc test. Two-way ANOVAs were used to test the effects of the experimental conditions on (1) the day diapausing egg production began, which was conservatively defined as the time for 5 % of the accumulative production of diapausing eggs, (2) total production of diapausing eggs through the course of the experimental time course, and (3) per capita diapausing egg production integrated over time. Additionally, the concentration of As^{3+} was measured by HG-AAS in dried rotifer samples obtained by filtering rotifer cultures. Samples of dry rotifer biomass were previously digested with 2 mL 67 % HNO_3 (Merck, Suprapure) and 0.6 mL 30 % H_2O_2 (Merck, Suprapure) in a PTFE vessel in a closed microwave device at 200 °C for 70 min. After evaporation of excess acid in the same microwave system, the resulting solution was filtered with Whatman 42 paper and transferred to a volumetric flask (10 mL). To diluted samples was added 10 mL of KI and 10 mL of concentrated HCl and allowed to stand for 20 min. Finally, it was up to volume with supra-pure water. Additionally, arsenic standards of 1, 5 and 10 μg L^{-1} were prepared, which were treated equally with KI and HCl. The curve was verified by reading a control sample of 3 μg L^{-1} and reading a certified reference material of known concentration was performed (368 μg L^{-1}). These measurements were made for the high food concentration cultures with arsenic added and the corresponding control. Samples were collected from vessels exposed for 2 days to the corresponding treatment (second day of experiment) and at the end of the experimental period after several generations exposed to treatments (18th day of the experiment).

Diapausing eggs obtained were classified from I to III in increasing deterioration state according to their viability-related features, as the proportion of egg occupied by the embryo and the integrity of protective covers (García-Roger et al. 2005). After scoring the eggs, hatching was induced by placing healthy (type I and II) eggs into 96-multiwell dishes with 200 μL of COMBO medium and incubating at 25 °C under constant white fluorescent illumination (150–170 μmol quanta $m^{-2}\ s^{-1}$) for up to 7 days. Dishes were checked every 24 h for hatchlings. Generalized linear models (GLM) were implemented in R v.3.2.2 statistical software (R Core Team 2015) and used for data analysis. The Poisson distribution and log link function were used for diapausing egg production, with arsenic, food and egg type as factors; the binomial distribution and logit link function were used for hatching proportion, with arsenic and food as factors.

Effect of arsenic on mixis ratio by brood

A life-table experiment was performed to assess variation of mixis ratio with maternal age in response to two factors: arsenic addition (with/without) × food level (low/high), with five replicates (females). Factor levels were as in previous experiments. Successive generations were monitored with the same procedure described above for the experiment on crowding cues, with cultures performed in wells of multiwell plates with 30 μL of COMBO medium, at the rather low density able to induce mixis, estimated in the experiment above. For each birth observed, mother age was recorded as 'brood' order, where brood means the set of offspring produced in the same day. Broods were numbered according to the days after mother maturity. Generalized linear models (GLM) (R Core Team 2015) with binomial distribution and logit link function were used for each generation obtained.

Results

Preliminary bioassays

The LC50 of As_2O_3 for *B. calyciflorus* was estimated as 44.5 ± 2.2 μg L^{-1} and EC50 for the microalga *P. subcapitata* growth was 370 ± 50 μg L^{-1}. Because a concentration 20 μg L^{-1} As_2O_3 did not produce significant mortality of rotifers (Fig. 1), in further experiments the nominal toxicant concentration used was 20 μg L^{-1} As_2O_3, as a sub-lethal concentration.

Induction of sexual reproduction by crowding cues

Correlation between mixis ratios in F_2 and F_3 within-experimental combinations was low and negative ($r = 0.155$; $t = 1.55$; d.f. $= 129$; $p < 0.05$). As no evidence of dependence between mother and offspring mixis ratios was observed, we used ANOVAs to analyze data in all experimental generations. In factorial ANOVA significant differences were found between mixis ratios obtained for different crowding levels were found for the F_1. Differences in mixis ratios were caused by crowding and food levels for the F_2, and F_3 (see results summarized in Table 1a). A significant increase in the mixis ratio was found when *B. calyciflorus* was exposed to crowding cues produced by 10 ind mL^{-1} and higher levels, regardless of the food concentration tested. This effect was observed in the F_2 and in F_3 (see results summarized in Table 1b). To guarantee an effective crowding cue regardless food level, experimental density to induce mixis was fixed at 30 ind mL^{-1} in the following experiments (Fig. 2).

Effect of arsenic on diapause eggs production in mass cultures

HG-AAS showed that As^{3+} concentration in the medium corresponding to a nominal concentration of 20 μg L^{-1} was 18.7 μg L^{-1} ± 0.2 (mean ± SD) averaged over time for the 10 samples to which arsenic was added. Arsenic slightly decreased population growth rate at high food level. Conversely, at low food concentrations a significantly higher growth rate than in control condition was obtained, showing an interaction between factors (Table 2, two-way ANOVA, d.f. $= 1, 16$, $F = 987.37$, $p < 0.001$). In the cultures where arsenic was added, after 2 days the arsenic concentration in rotifers was of 9.52 ppb (in 43 mg of rotifer sample), while after 18 days this value was

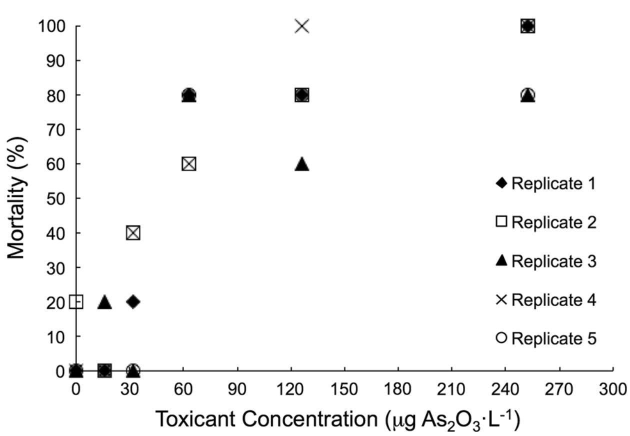

Fig. 1 Concentration–response curve of As_2O_3 on *B. calyciflorus* mortality (%). Replicates are shown separately

Table 1 Resume of significant results obtained in (a) factorial ANOVAs on mixis ratio of *Brachionus calyciflorus* in generations F_1, F_2, and F_3 and (b) Student–Newman–Keuls analysis method for multiple comparisons by crowding level factor in generations F_2 and F_3

(a) Source of variation	d.f.	F	p
F_1			
Crowding level	4, 40	3.8	0.01
F_2			
Food level	1, 40	6.8	0.013
Crowding level	4, 40	36.5	<0.001
F_3			
Food level	1, 40	12.7	<0.001
Crowding level	4, 40	28.1	<0.001

(b) Crowding level	q	p
F_2		
10 ind mL^{-1}	6.1	<0.001
20 ind mL^{-1}	9.8	<0.001
40 ind mL^{-1}	14.4	<0.001
F_3		
10 ind mL^{-1}	5.1	0.002
20 ind mL^{-1}	9.1	<0.001
40 ind mL^{-1}	12.6	<0.001

279 ppb (in 121 mg of rotifer sample), which was approximately 30 times the initial value. The corresponding values for rotifers in control cultures were 0.018 ppb (in 88 mg of rotifer sample) and 0.025 ppb (in 230 mg of rotifer sample).

Diapausing egg production was significantly greater and earlier than the control at low food level, while at high levels of food were no differences caused by arsenic, although a delay in the beginning of diapausing egg production was observed when the toxicant was present (Table 3). This is reflected by significant interaction effects on the initiation of diapausing egg production (two-way ANOVA, d.f. $= 1, 16$, $F = 25.0$, $p < 0.001$) and on total production of diapausing eggs (two-way ANOVA, d.f. $= 1, 16$, $F = 38.2$, $p < 0.001$). A delayed investment was obtained without toxicant and low food availability. Total production of diapausing eggs of investment was significantly greater in the treatment with arsenic and low food than in all other treatments (Table 3). Figure 3 shows per capita diapausing egg production. Consistently with the pattern detected by the ANOVA on the initiation of diapausing egg production and total diapausing egg production, the treatment with arsenic caused a much higher per capita investment in diapause (two-way ANOVA, d.f. $= 1, 16$, $F = 26.7$, $p < 0.001$).

The quality of eggs produced depended on the mother's environment. Regardless of food level, without arsenic

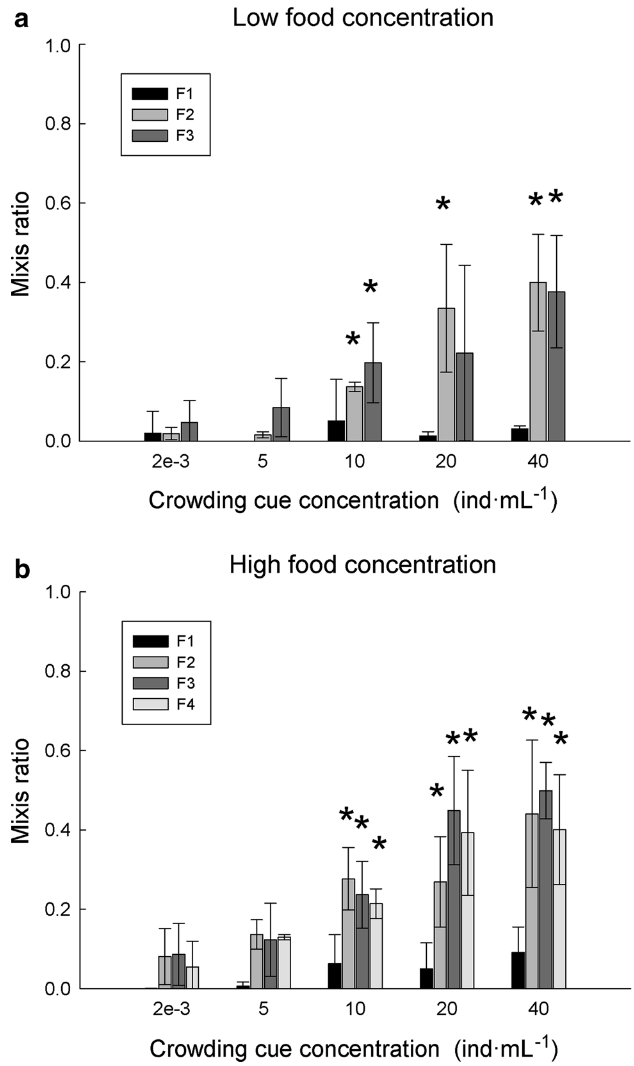

Fig. 2 Mixis ratio of *B. calyciflorus* over generations at **a** low food level, 0.5×10^6 cells *P. subcapitata* mL^{-1}, and **b** high food level, 2.5×10^6 cells *P. subcapitata* mL^{-1}, produced in a gradient of conspecific crowding cues (mean ± 95 % CI). *Asterisks* show differences relative to control

addition the highest quality eggs (type I) were produced in greater proportion, while with toxicant and low food mostly poor quality eggs (type III) were produced (Fig. 4). Consistently, according to the GLM analysis the production of the different egg types depended on arsenic addition ('egg type × arsenic addition' interaction; Chi square $p < 0.001$) and on food level ('egg type × food level' interaction; Chi square $p < 0.001$).

Finally, according to the GLM analysis the hatching success was dependent on arsenic addition (Chi square $p < 0.001$) and food concentration (Chi square $p < 0.001$). Higher hatching success was observed in eggs produced in toxicant-free medium, regardless of whether there was low (*t* test, $t = 3.175$, d.f. = 4, $p < 0.005$) or high food level (t-test, $t = 4.791$, d.f. = 4, $p = 0.009$) (Table 2).

Effect of arsenic on mixis ratio by brood

Sexual offspring were not obtained in F_1 generation. The results showed an effect of food (GLM, Chi square $p = 0.01$) and arsenic (GLM, Chi square $p < 0.001$) on mixis produced by the F_2. Mixis ratio produced by the F_3 depended on food (GLM, Chi square $p = 0.001$) and on the interaction of factors (Chi square $p < 0.01$). Although arsenic addition produced a tendency to increase the mixis ratio in the intermediate and last broods of the F_2, the effects were not significant between broods in either generation (Fig. 5).

Discussion

Addition of sublethal concentrations of arsenic modified the pattern of diapausing egg production observed in the rotifer *B. calyciflorus*. Regardless of food availability, with arsenic addition investment in diapausing eggs peaked earlier and higher than in non-toxicant conditions. This might be considered as an escape strategy in response to risky conditions imposed by increased toxicity. Nevertheless, the eggs produced in large amount in medium with arsenic were mostly low quality, as shown by their morphological type; in addition, the apparently healthy eggs had lower hatching success. Thus, arsenic had a physiological cost with detrimental effect on a fitness component, i.e., egg quality. In cyclical parthenogenetic organisms where sex is associated with diapause, as in monogonont

Table 2 Daily population growth rates (mean ± 95 % CI) and hatching success (% ± 95 % CI) of diapausing eggs produced of rotifer *Brachionus calyciflorus* exposed to As_2O_3 at 20 mg L^{-1} and at low (0.5×10^6 cells mL^{-1}) and high (2.5×10^6 cells mL^{-1}) density of *Pseudokirchneriella subcapitata* as food

Food concentration	Arsenic	Population growth rate ± 95 % CI (d^{-1})	Hatching success (% ± 95 % CI)
Low	Not added	0.33 ± 0.01	66 ± 16
	Added	0.788 ± 0.004	40 ± 4
High	Not added	0.91 ± 0.03	85 ± 10
	Added	0.876 ± 0.01	61 ± 1

Table 3 Starting of the diapausing egg production and total diapausing egg production by *B. calyciflorus* exposed to medium control and with As_2O_3 (20 μg L^{-1}), with low (0.5 × 10^6 cells mL^{-1}) and high (2.5 × 10^6 cells mL^{-1}) density of microalga *P. subcapitata* as food. Mean values ± 95 % CI are shown

Food concentration	Arsenic	Starting of the diapausing egg production (d)	Total diapausing egg production (L^{-1})
Low	Not added	8 ± 1.1	50.2 ± 5.7
	Added	3.2 ± 0.4	195 ± 14.5
High	Not added	5.8 ± 0.4	117.4 ± 6.5
	Added	7.6 ± 2.3	110.4 ± 25.1

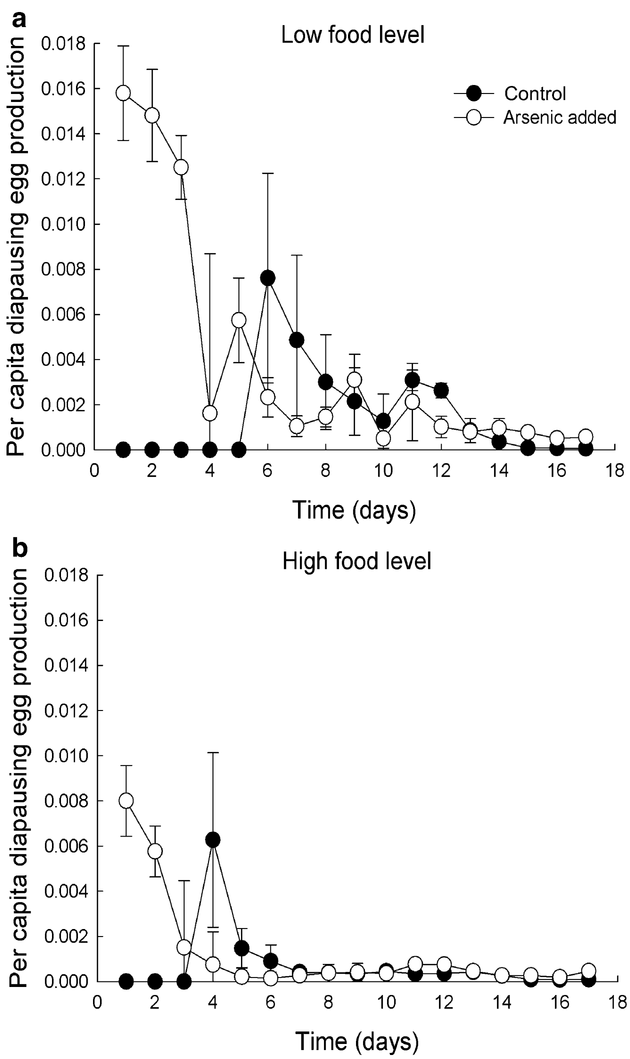

Fig. 3 Dynamics of per capita diapausing egg production (mean ± 95 % CI) by *B. calyciflorus* in cultures with arsenic addition and control without arsenic added. **a** In low food level, 0.5 × 10^6 cells *P. subcapitata* mL^{-1}, and **b** high food level, 2.5 × 10^6 cells *P. subcapitata* mL^{-1}

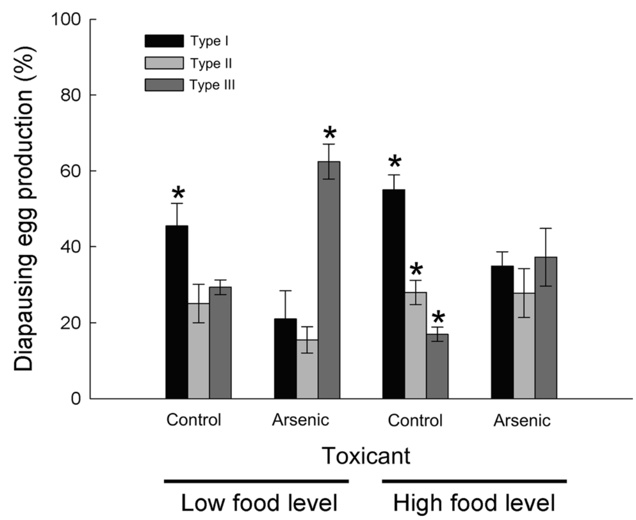

Fig. 4 Percentage of diapausing eggs, categorized by deterioration state (I–III), produced by *B. calyciflorus* in cultures combining: (1) with arsenic added, and a control without arsenic added, and (2) two food levels: low (0.5 × 10^6 cells *P. subcapitata* mL^{-1}) and high (2.5 × 10^6 cells *P. subcapitata* mL^{-1}). Asterisks show significant differences between egg types

rotifers, a trade-off exists between fast clonal proliferation of the current population (short-term growth) and population persistence through diapausing eggs produced sexually (long-term persistence; Serra and Snell 2009; Gilbert and Schröeder 2004). Frequently, not all individuals of a clone are involved in diapausing egg production (Fussmann et al. 2007), nor do all diapausing eggs leave diapause when suitable conditions in the water column resume, so that diapause is under bet-hedging strategies to spread risk. Local conditions are expected to drive the fine-tuning of the optimal strategy for this trade-off. Thus, the question arises whether the pattern of investment in diapause observed in our experiments is optimum in an environment with arsenic, or if rather arsenic presence in water bodies disturbs the optimal allocation of offspring going into diapause. Heavy metal concentration in general and arsenic concentration in particular, may produce selective pressure on rotifer populations, as has been suggested from *Daphnia* egg banks in historically metal polluted sediments (Rogalski 2015). First, volcanic, geothermal activity, in conjunction with evapo-concentration in arid zones should have an effect on environmental concentration of arsenic;

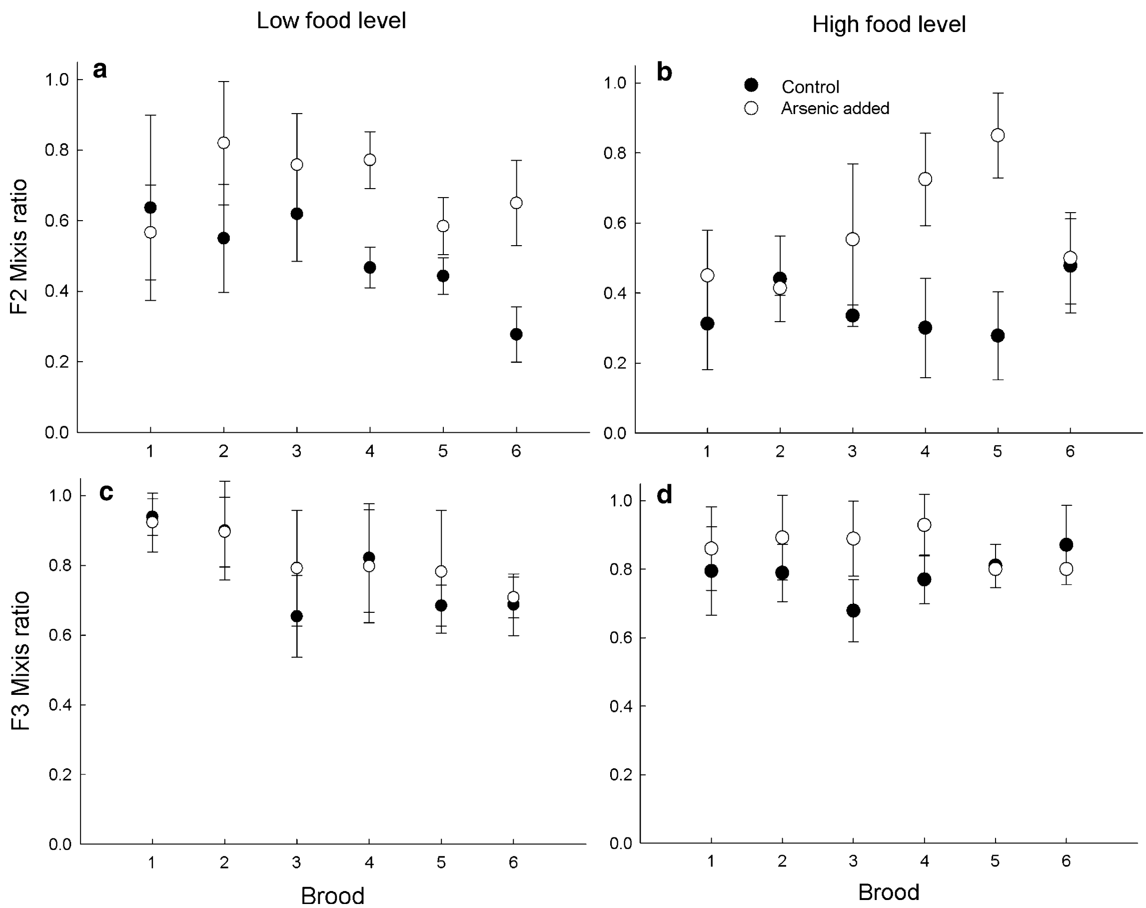

Fig. 5 Mixis ratio of *B. calyciflorus* produced in successive broods at **a**, **c** low food level (0.5×10^6 cells *P. subcapitata* mL^{-1}), and **b**, **d** high food level (2.5×10^6 cells *P. subcapitata* mL^{-1}) with arsenic added, and a control without arsenic added (mean ± 95 % CI). Upper row, F_2 generation; lower row, F_3 generation

second, the mining industry has been developed since the XIX century in the region (Dittmar 2004; Salvarredy-Aranguren et al. 2008). As cyclically parthenogenetic zooplankters evolve rapidly (Fussmann 2011; Declerck et al. 2015), it is plausible to conjecture adaptation of diapause stage production patterns under high heavy metal fluctuations. High heavy metal concentrations are known to affect the rotifer community (Cecchine and Snell 1999; Weisse et al. 2013). Additionally, the diapause pattern observed in different organisms as response to arsenic might be a general response to stressors (Aruda et al. 2011). Nevertheless, a full assessment of fitness including the performance of individuals hatched from surviving eggs would be required to get further insight on this question.

Our results suggest that sexual investment initiated by crowding could be enhanced in highly stressed environmental conditions. Previous research has highlighted the ecological and evolutionary relevance of the responsiveness of rotifers to environmental hazard cues. Behavioral (Gilbert 2014) and morphological (Aránguiz-Acuña et al. 2010) responses in *Brachionus* rotifers are expressed earlier in conditions of increased predation risk. In the case of sexual reproduction, optimal timing of initiation is likely dependent on several factors, such as habitat deterioration (e.g. Serra and Carmona 1993; Carmona et al. 1993), resource demand (Snell and Boyer 1988; Serra and King 1999) and male–female encounters (Snell and Garman 1986). Sex initiation is density-dependent in the genus *Brachionus*, triggered by a threshold concentration of infochemical released by the organisms themselves. The chemical environment may affect the ability of individuals to detect or to respond to the infochemicals of ecological relevance. This phenomenon, known as info-disruption, has been observed in the inhibition or inappropriate induction of morphological defenses in zooplankton groups (Hanazato 1996; Sakamoto et al. 2006). Although our results do not demonstrate directly a displacement of the density threshold for sex induction, they showed a response in the timing of sex initiation, associated with the presence of arsenic in the medium.

Diapausing egg quality, measured as embryo size within the egg and hatchability, has been linked to biotic and abiotic factors in *Brachionus*. Higher hatching success has been observed in diapausing eggs produced both under optimal salinity (Gabaldón et al. 2015)—i.e. favorable conditions—and under an interspecific competition scenario during their production (Aránguiz-Acuña et al. 2015)—i.e. unfavorable conditions. These contrasting results stress that an adverse condition can result in either a low quality of diapausing eggs, as a trivial effect of bad conditions, or in an effort to produce high quality eggs as an escape response towards the future, likely with better conditions. Although our results point to the former scenario, greater production of eggs with addition of arsenic suggests a resource allocation effort to diapause, i.e. to long-term survival.

Previously, effects of pesticides and sedimentary metals have been observed on development and hatching success of the diapausing eggs of cladocerans *Daphnia* (Navis et al. 2013, 2015; Rogalski 2015). Additionally, a field study showed metals were accumulated by *Daphnia* ephippia (Wyn et al. 2007). These antecedents suggest that toxicants are incorporated to diapausing stages from the medium, and that even though diapausing stages facilitate the survival of aquatic organisms under extreme conditions due to suppressed metabolism, there is contradictory evidence about the resistance to toxic agents in zooplankton embryonic development when embryos are still inside protective membranes. While Alekseev et al. (2010) showed great capability of diapausing eggs to resist severe pollution events in several zooplankton groups, Navis et al. (2015) suggested that the cladoceran ephippium offers limited protection against pesticides dissolved in the medium. Moreover, pesticide bioconcentration in the eggs increases with exposure time during the embryonic development (Navis et al. 2015). Although we could not assess the concentration of arsenic in the diapausing eggs, we observed a very high accumulation of toxicant in organisms exposed to constant arsenic concentration after several generations. Alvarado-Flores et al. (2012) found high and fast deposits of granules of Pb in the mastax and, most relevant, in the vitellarium of *B. calyciflorus* experimentally exposed to that metal. Thus it can be proposed that arsenic accumulates transgenerationally not only in females, but also in diapausing eggs, and thus accumulative effects of mothers exposed to metals in the environment may play a role in the hatching success and in the hatchlings performance (Rogalski 2015).

Sensitivity to arsenic, measured through LC50 assessment, was in the range observed in rotifers exposed to heavy metals such as Cd and Pb (Arias-Almeida and Rico-Martínez 2011). Although our study was focused on arsenic, at concentrations currently occurring in some natural ponds (Pell et al. 2013), most likely these animals are not exposed to a single heavy metal, which may influence in the rotifer response to these toxicants (Jara and Aránguiz-Acuña 2013). Ríos-Arana et al. (2005) showed contrasting effects on induction of heat shock protein HSP60 by the rotifer *Plationus patulus* when was exposed to arsenic alone and to arsenic mixed with other heavy metals. They, concluded that rotifer response depends on the type and number of elements present in the medium. Therefore, responses observed in this study could be considered as informative for future evaluations, in which complex experimental conditions or field conditions could be included.

B. calyciflorus reached higher population growth than in the control condition under unfavorable experimental conditions, i.e. low food and toxicant added. Density-dependent compensation has been observed in previous toxicity assays, which would buffer negative effect of toxicants (Forbes et al. 2001; Barata et al. 2002), and even results in a positive synergistic impact on growth rate (Raimondo 2013). The phenomenon of hormesis has been frequently invoked, by which an organism's performance is stimulated at low levels of exposure to agents that are toxic at high levels (Forbes 2000). This argument has already been used to explain the response of high growth rates (Moreira et al. 2015) and other beneficial behavioral traits (Chen et al. 2014) in rotifers exposed to low concentrations of toxicants. Regardless of the mechanisms driving this trend, our results suggest that significant toxicological effects may be observed in parameters related to the production and quality of diapausing eggs, although gross endpoints, such as population growth rate, are insensitive to toxicants. In rotifers there is broad evidence that the sexual phase can be more sensitive to environmental conditions, specifically exposure to toxicants, than the asexual phase (Snell and Carmona 1995; Snell et al. 1999; Preston and Snell 2001; Marcial et al. 2005). Our results are in agreement with those antecedents, but in addition this study provides interesting information about the adaptive value of the diapause strategy against adverse conditions. Therefore, our results suggest that attributes involved in diapause should be progressively considered in ecotoxicological assessment and as environmental markers (Wyn et al. 2007) over different temporal scales because of its ecological and evolutionary implications on lake biodiversity.

Acknowledgments This study was supported by FONDECYT Grant 11130653 to A. A.-A. The authors thank to Pablo Pérez and undergraduate students Jazmín Hidalgo, Noelia Álvarez and Ana de la Fuente for their technical assistance in the laboratory.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest

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