

Isolation by sugar flotation has no direct effect on the hatching success of zooplankton resting eggs

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ABSTRACT

Zooplankton in temporary waters produces resting stages to survive recurrent dry periods. Branchiopod crustaceans (i.e., cladocerans, large branchiopods) overcome these periods in the form of resting eggs buried in the sediment. Examining the diversity in the resting egg banks allows for a more accurate estimation of biodiversity of aquatic ecosystems than looking only at the active communities. The isolation of resting eggs from the sediment may be achieved by the sugar flotation method, which usually results in higher density and diversity than untreated samples (i.e., incubated in the sediment). We tested the effect of sugar isolation and centrifugation on the hatching success of resting eggs already isolated from sediment in order to reveal any direct effects on hatching success. We used four different branchiopod species, *Daphnia magna*, *Moina brachiata*, *Branchinecta orientalis*, and *Triops cancriformis*. Although we hypothesised that osmotic stress caused by sugar and centrifuging influence the hatching success either positively (e.g., faster activation as a response to osmotic changes) or negatively (destroyed by centrifugation), we found no significant difference either in the timing or rate of hatching between centrifuged and non-centrifuged eggs. Once the eggs are exposed to light and/or oxygen availability by being removed from the sediment, the centrifugation process does not have any additional effect on their hatching. Regardless of treatment, we found a significant difference in the hatching timing in the two major groups, with large branchiopods hatching earlier than cladocerans. We found that the sugar flotation method itself does not influence the hatching fraction of branchiopod resting eggs (implying no adverse effect on their viability) and its success in enhancing hatching can be attributed to its efficiency in removing eggs from the sediment.

Key words: Egg bank; sugar flotation method; large branchiopods; Cladocera; resting eggs.

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INTRODUCTION

Temporary aquatic habitats include very diverse ecosystems regarding size, shape, hydroperiod and chemistry. They are widespread throughout the world, being the most numerous in arid and semi-arid climatic regions (Brendonck, 1996). Their main common characteristic is that they are periodically unfavourable for many aquatic organisms as a consequence of drought (Schwartz and Jenkins, 2000). In extreme cases, they can remain dry for several years, even decades. Additionally, oxygen depletion and extreme temperatures can also develop at certain periods of the year (Brendonck and De Meester, 2003). During drought, sediment is exposed to strong solar radiation and high temperatures (Dumont and Negrea, 2002). Since this environment is highly unfavourable for many aquatic organisms, permanent residents possess specific life cycle adaptations, like reduced length of life and resting stages resistant to drying out.

Branchiopods (fairy shrimps, tadpole shrimps, clam shrimps and water fleas) are frequent members of temporary aquatic communities. Accordingly, their resting eggs are resistant to a wide range of environmental conditions

(e.g., high insolation, extreme temperatures) (Brendonck and De Meester, 2003; Brock *et al.*, 2003; Havel and Shurin, 2004; Radzikowski, 2013). This group developed bet-hedging strategy as a response to the unpredictable environment. As only a fraction of the resting eggs stored in the egg bank hatches during one inundation, this protects the local populations from extinction in case of abortive hatching (Hildrew, 1985; Brendonck, 1996; Simovich and Hathaway, 1997; Pincheel *et al.*, 2013; Wang and Chou, 2015) and lessens population inbreeding by spreading generations over several years (Rogers, 2014, 2015). Dormant egg banks are not only important for bet-hedging, but they also contribute considerably to the temporal and spatial variation in active community composition (Brendonck and De Meester, 2003). Finally, dormant egg banks capture the total species set of habitats well (by being a footprint of all seasonally different active communities), which makes them an appropriate tool for biodiversity assessment (Vandekerckhove *et al.*, 2005b). These latter features make egg banks important not only for temporary, but also for all permanent waterbodies.

In general, egg hatching is triggered by a combination

of several environmental factors that most probably reflect the current quality of the habitat for growth and reproduction. The most important factors are osmotic pressure, oxygen, temperature and light exposure, but their significance varies between climatic regions (Brendonck, 1996; Brendonck and De Meester, 2003). For example, it was suggested that in the temperate zone, the most important triggers are temperature and photoperiod (Vandekerkhove *et al.*, 2005a). Brown and Carpelan (1971) suggested that in arid regions, osmotic pressure (as indicator of salinity) and oxygen could be considered as the most important factors for hatching triggers, while in temperate regions, temperature and oxygen levels could be more indicative to inhabitants of temporary ponds.

As the dormant eggs are stored in the sediment, this makes them generally difficult to obtain and use in applied studies. Brine solution can be used to increase the number of floating eggs in a diluted sediment sample and facilitate their separation (Rogers, 2014). The sugar flotation method, introduced to accelerate and enhance the isolation success of dormant invertebrate eggs, was first applied on marine zooplankton (Onbé, 1978), and later modified by Marcus (1984; 1990). Onbé (1978) tested the success of egg separation through centrifuging on one cladoceran and one copepod species and found that centrifugation caused fast and effective separation of the eggs from the sediment. He also reported that centrifuging does not influence egg viability of these two marine species. Additionally, it was reported that sonication does not affect egg viability either (Marcus, 1984; De Stasio, 1989). However, in all these reports, it was not described in detail under which conditions untreated (control) eggs were incubated and there is only an indication that this method had no direct effect on hatching success of zooplankton eggs. In Vandekerkhove *et al.* (2004), the sugar flotation method was explicitly tested for the first time. In this study, the hatching of dormant eggs was compared between treatments with and without isolation with the sugar flotation. Isolation increased the overall hatching success and lowered activation time (the time needed for the emergence of hatchlings). In a similar study, hatching rates were also found to be significantly higher when the sugar flotation method was applied in the case of cladocerans and ostracods, while the overall number of anostracan hatchlings was higher in untreated samples (Lieverink *et al.*, 2014). The latter two studies indicate that there can be pronounced differences between the hatching successes of zooplankton groups related to the applied incubation method. However, it remains unclear how the sugar flotation method itself affects individual zooplankton species as resulting eggs were always compared to unisolated eggs, *i.e.* eggs incubated with sediment.

The effect of centrifugation in sugar solution, as described in the sugar flotation method (Onbé, 1978; Mar-

cus, 1990) was never explicitly tested on individual freshwater species and the hatching rates of treated and untreated eggs separated a priori from the sediment was never compared. Hence, it remains unclear, whether the sugar flotation method has a direct influence on the hatching success of crustacean zooplankton resting eggs (*e.g.*, increasing the rate through osmotic stress or by reducing viability through centrifugation) or if it acts only indirectly through enhancing the isolation from the sediment. In this study, we present a comparison of hatching rate and hatching timing with and without applying the sugar flotation method on previously isolated eggs.

METHODS

We chose four branchiopod species for the experiments, representing their two main groups of temporary waters, cladocerans and large branchiopods. We used three widely distributed branchiopod species, the notostracan *Triops cancriformis* (Bosc 1801), and among cladocerans, the large-bodied *Daphnia magna* Straus, 1820, along with another smaller sized temporary water species, *Moina brachiata* Baird, 1850. The anostracan *Branchinecta orientalis* G. O. Sars, 1903 is frequently found in soda pans of the Carpathian Basin (Horváth *et al.*, 2013), and these four species occasionally co-occur in the egg banks of the soda pans (Horváth, *unpublished data*). We collected sediment from soda pans of the National Park Neusiedler See–Seewinkel (Austria) that are known to host these species in their egg bank in large numbers: Östliche Fuchslochlacke, for *D. magna* and *T. cancriformis* eggs; Lange Lacke, for *M. brachiata* eggs; and Oberer Stinkersee for *B. orientalis* eggs.

Material was stored in the dark at 4°C for a year. The sediment was sonicated for 30 s and sieved through a 250 µm mesh to isolate the eggs. We separated intact and healthy-looking eggs from the material retained in the sieve under a stereo microscope. Half of the separated eggs (treated group) was put into tubes containing sugar solution (1 kg of sucrose dissolved in 1 L of distilled water) and centrifuged at 3000 r min⁻¹ for 3 min in accordance with the Onbé-Marcus sugar flotation method (Onbé, 1978; Marcus, 1990). After centrifuging, eggs were rinsed with distilled water through a sieve (30 µm mesh size) to remove the sugar residues and incubated for hatching. The second group (untreated group) was immediately incubated for hatching after removal from the sieved sediment. We put 20 eggs/ephippia of *B. orientalis*, *T. cancriformis*, *D. magna* or *M. brachiata* into the experimental vials (in case of *D. magna*, 40 eggs per vial, since each ephippium contains two eggs). This was replicated three times per treatment.

One hundred mL of artificial soda water (NaHCO₃ dissolved in distilled water with a concentration of 0.4 g L⁻¹) of a conductivity of 500 µS cm⁻¹ (Boros *et al.*, 2014) was

added to each hatching vial. Vials were placed in a walk-in chamber with light set for 16 h *per* day and constant temperature of 18°C, conditions considered optimal for hatching cladocerans (Vandekerkhove *et al.*, 2005a). The experiment ran for two weeks. We checked the vials daily for emerging juveniles, which were removed. The number of hatchlings per vial (*i.e.*, hatching rates) and the day of hatching (hatching timing) for each individual were recorded.

For testing the treatment effect on hatching rates, we performed an independent two-group t-test for each of the species. Normal distribution of hatching rates was tested with Shapiro-Wilk tests within each treatment group per species. In case of normality, homogeneity of variances was also tested with an F test. Homogeneous variances were found in *D. magna*, *M. brachiata*, *B. orientalis*, while variances were non-homogeneous in *T. cancriformis* (here we used the Welch approximation to the degrees of freedom).

Two-way mixed model ANOVAs were performed to test for the differences in the hatching timing among the

treatments (fixed), correcting for the replicates (random factor) in all tested species. The effect of random factor was never significant ($P > 0.05$). To test for taxonomic group related differences in hatching timing, we used a mixed model ANOVA with taxonomic group (cladocerans and large branchiopods) as the fixed, and treatment and species as random factors. Analyses were performed with the statistical software R (R Development Core Team, 2009).

RESULTS

Hatching timing

We found no significant differences in hatching timing between treated and untreated eggs. *Daphnia magna* hatched on day 6 both in treated (mean \pm SE: 6.11 ± 0.27 ; means reported in the text are calculated for previously obtained means of the replicates) and untreated (6.17 ± 0.64) samples ($F_{(1,38)} = 22.01$; $P = 0.13$; Fig. 1), while *Moina brachiata* hatched on day 5 both in treated (5.15 ± 0.78) and untreated (5.33 ± 1.05) samples ($F_{(1,21)} = 2.45$; $P = 0.36$; Fig. 1).

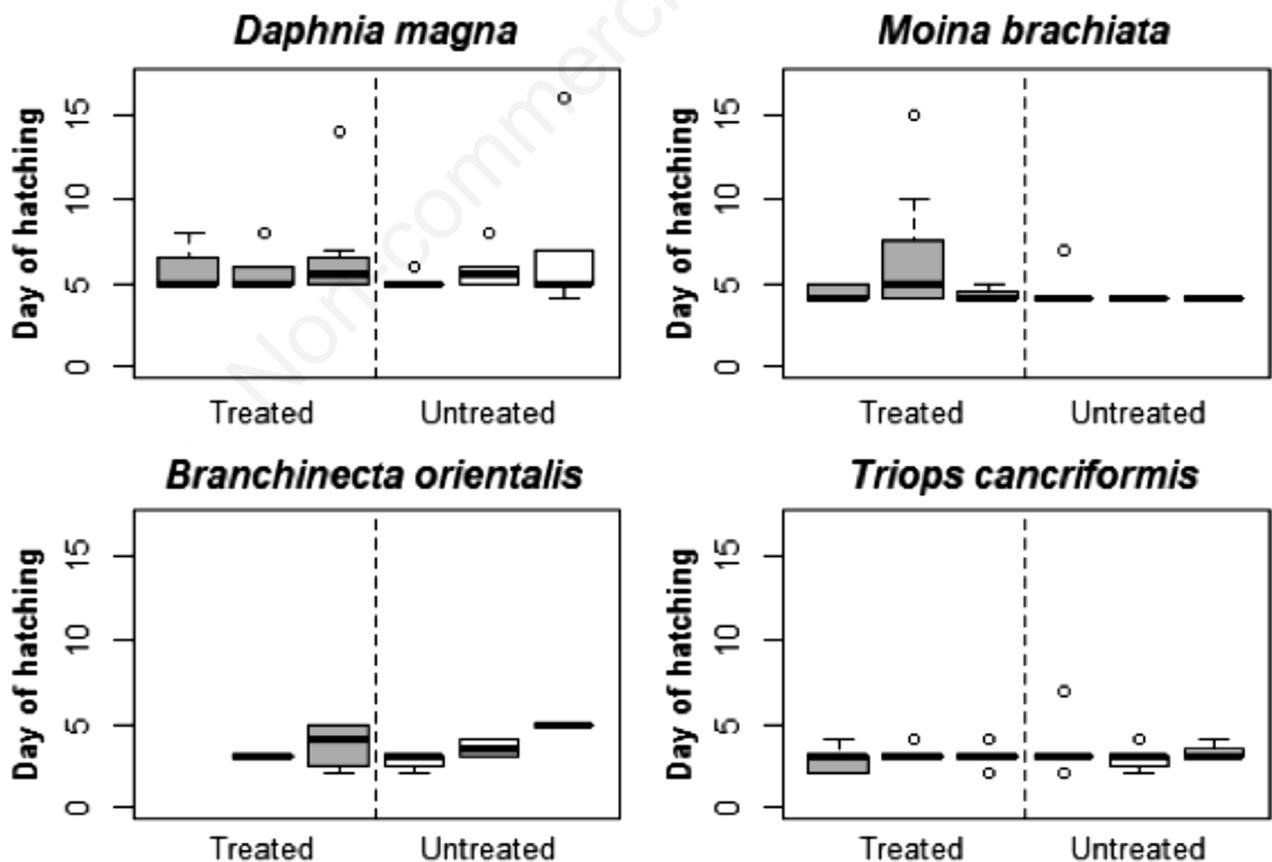


Fig. 1. Hatching timing (day of hatching) in individuals of *Daphnia magna* ($F_{(1,38)} = 22.01$; $P = 0.13$), *Moina brachiata* ($F_{(1,21)} = 2.45$; $P = 0.36$), *Branchinecta orientalis* ($F_{(1,7)} = 131.4$; $P = 0.06$), and *Triops cancriformis* ($F_{(1,51)} = 0.27$; $P = 0.69$) for treated and untreated eggs.

In *D. magna*, most (>95%) hatchlings emerged between days 4-8 of the experiment, while *M. brachiata* (100%) hatched between days 4-7. *Branchinecta orientalis* hatched between day 3 and 4 in treated (3.37 ± 0.37) and untreated (3.72 ± 0.68) samples ($F_{(1,7)}=131.4$; $P=0.06$; Fig. 1). Eggs of *Triops cancriformis* hatched on day 3 on average both in the treated (2.96 ± 0.14) and untreated (3.17 ± 0.15) samples ($F_{(1,51)}=0.27$; $P=0.69$; Fig. 1). All *B. orientalis* individuals hatched between the 2nd and 5th day of the experiment, while *T. cancriformis* mostly (>98%) hatched between the 2nd and 4th day.

Cladocerans generally hatched later than large branchiopods. Mean hatching (regardless of treatment) in cladocerans was day 5.65 ± 0.29 , while in large branchiopods it was two days less (3.14 ± 0.10). This difference was also significant in the ANOVA (fixed factor of taxonomic group: $F_{(1,2)}=22.79$, $P=0.04$). In addition, the first cladoceran hatchling emerged on day 4, while the first large branchiopod nauplius only appeared on day 2.

Hatching rates

There was no significant difference in hatching rates between the treated and untreated eggs for any of the studied species. Average hatching rates in *D. magna* were slightly higher in untreated samples (20 ± 3.82 vs $15 \pm 3.82\%$), but this difference was not significant (t -test, $t=-0.93$; $P=0.41$; Fig. 2). In *M. brachiata*, the hatching rates were somewhat higher in treated samples (but again not significant, t -test, $t=1.15$; $P=0.32$; Fig. 2), with mean values $25 \pm 5.77\%$ in treated and $16.67 \pm 4.41\%$ in untreated samples.

The anostracan *B. orientalis* showed a similar, but not significant tendency as *D. magna*, with a mean hatching rate of $8.33 \pm 6.00\%$ in the treated samples and $10 \pm 2.89\%$ in untreated samples (t -test, $t=-0.25$; $P=0.82$; Fig. 2). *T. cancriformis* showed overall higher hatching rates than the other species and a tendency similar to *M. brachiata*. In the treated samples, *T. cancriformis* mean hatching rate

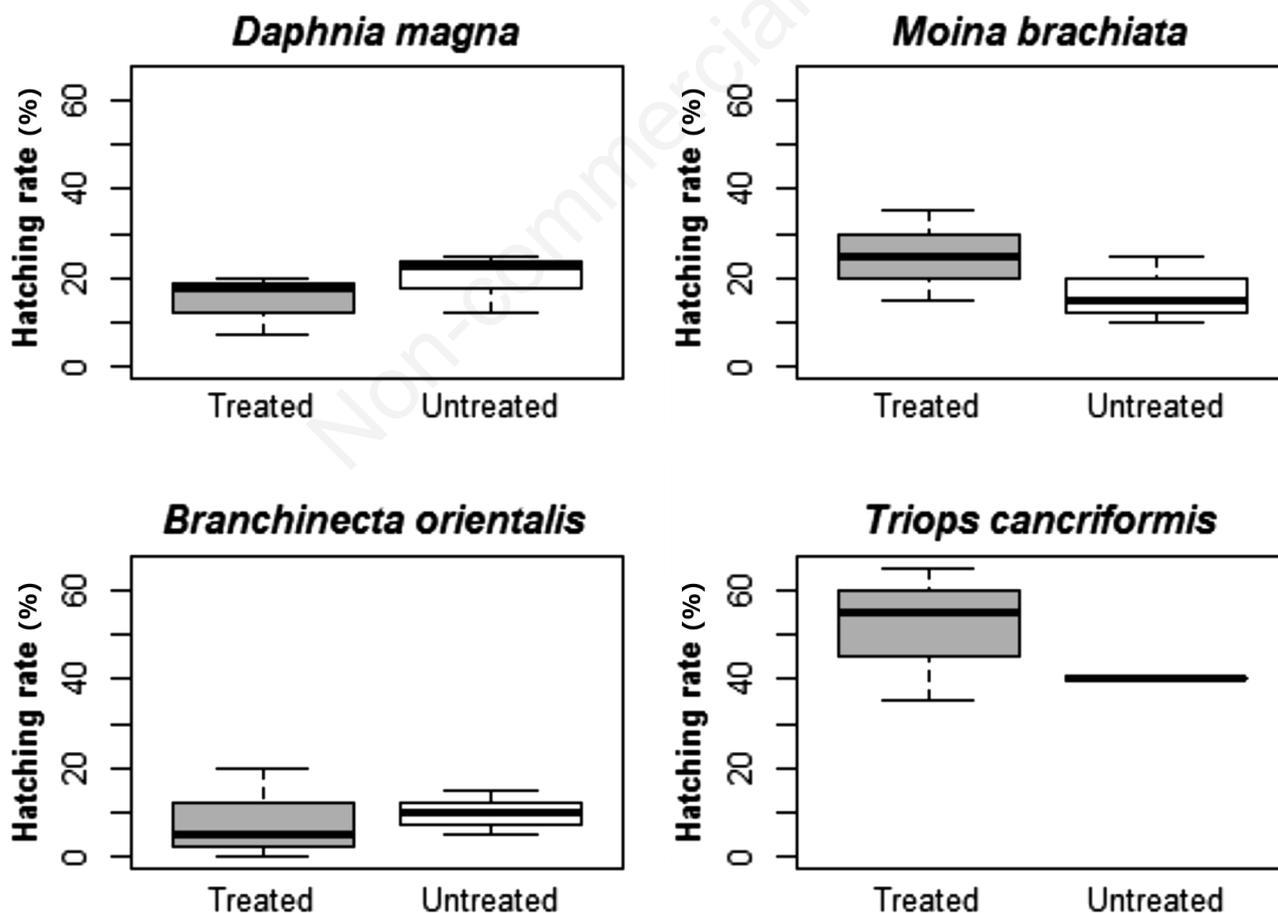


Fig. 2. Hatching rates of *Daphnia magna* ($t=-0.93$; $df=4$, $P=0.41$), *Moina brachiata* ($t=1.15$; $df=4$, $P=0.32$), *Branchinecta orientalis* ($t=-0.25$; $df=4$, $P=0.82$), and *Triops cancriformis* ($t=1.32$; $df=2$, $P=0.32$) for treated and untreated eggs.

was $51.67 \pm 8.82\%$, while it was $40 \pm 0.00\%$ in the untreated samples (t -test, $t=1.32$; $P=0.32$; Fig. 2).

DISCUSSION

Vandekerkhove *et al.* (2004) previously reported that hatching rates were higher and hatching was more rapid using the sugar flotation method as opposed to the direct incubation of eggs without isolating them from the sediment. They argued that the isolation procedure itself may have contributed to the results (*i.e.*, direct effect), but changes in other environmental factors, such as exposure to light or oxygen availability after isolation could have also had indirect effects. Our study demonstrated that the efficiency of the sugar flotation method is attributable to environmental factors.

Many studies report the significance of light exposure for hatching of various zooplankton taxa (reviewed in Brendonck, 1996), like the anostracan *Streptocephalus macrurus* Daday, 1908 (Mitchell, 1990), the notostracan *Triops longicaudatus* (LeConte, 1846) (Scott and Grigarick, 1979) and the cladoceran *Daphnia* sp. (Schwartz and Hebert, 1987). Oxygen availability also proved to be important for the hatching success of various zooplankton groups (Brown and Carpelan, 1971; Lutz *et al.*, 1992; Roman *et al.*, 1993). These records are consistent with claims that hatching success is strongly reduced when anostracan (Brendonck *et al.*, 1998; Shardin *et al.*, 2015) and notostracan (Scott and Grigarick, 1979) eggs are buried in the sediment. It is also suggested that light-triggered hatching is an adaptive mechanism contributing to bet-hedging. As it only induces the hatching of a fraction of eggs, by having little or no effect on the eggs covered by sediment, this limits egg bank depletion in case of abortive hatching (Brendonck *et al.*, 1998).

Our results show that osmotic pressure caused by sugar and centrifuging does not have significant influence on branchiopod hatching timing and rates. Our results suggest that once the eggs had been removed from the sediment, they were exposed to better light and (very likely) oxygen conditions, that were more important factors in the regulation of hatching than the isolation method itself.

Hatching was synchronised in the treated and untreated samples within the same species. Schwartz and Hebert (1987) demonstrated that, at a temperature same as in our experiment (18°C), *Daphnia* eggs started to hatch five days after inundation. Our results for the day of hatching in *Branchinecta orientalis* (mean: ~ 3 days, earliest hatchlings emerging on day 2) were consistent with those obtained by Atashbar *et al.* (2014), who tested the hatching success of three anostracan species, including *B. orientalis*, at different salinities and temperatures. Atashbar *et al.* (2014) also found that anostracans start to hatch two days after inundation at lower salinity and temperature.

We found that cladocerans hatched later than large

branchiopods. This difference in the hatching timing can be related to the embryonic development within the egg as anostracans and notostracans emerge from the eggs in larval stage (as nauplii), while cladocerans appear as miniature adults (Dumont and Negrea, 2002). Additionally, once the eggs are activated in large branchiopods, there is no further division of the cells, but the existing cells of an embryo rapidly differentiate into a nauplius, which is different in cladocerans, where both cell division and differentiation take place (Dumont and Negrea, 2002).

Regardless of treatment effect, there was a large difference among hatching rates between species. *Triops cancriformis* had the highest hatching rate (mean for treated and untreated samples together was 45.83%), followed by *Moina brachiata* (mean 20.83%) and *Daphnia magna* (mean 17.5%), and the lowest observed hatching rate was in *B. orientalis* (mean 9.17%). In a study with several species of genus *Branchinecta*, the hatching rates were even lower than in our case, especially in soil samples ($0\text{--}4\%$), while hatching rates in eggs recovered from waterbirds were higher, but still relatively low ($0\text{--}14\%$) (Rogers, 2014). In their review, Brendonck and De Meester (2003) similarly found that anostracans frequently have hatching rates below 20% . Moreover, wide intraspecific differences in hatching success were found previously even within the same populations of *D. magna* (De Meester and De Jager, 1993) and *Daphnia pulicaria* (Cáceres and Tessier, 2003).

We suggest that the hatching rate results in our study, ranging from 0 to 65% , were not influenced by the separation solution, but were an effect of dormancy itself. Briski *et al.* (2013) reached the same conclusion after obtaining similar hatching rates in their experiment where they compared two separation methods, sugar flotation and colloidal silica sol Ludox HS 40. The generally low hatching rates could be the consequence of bet-hedging strategy. Due to the unpredictable nature of temporary waters, deciding whether to stay dormant or to hatch, represents an important decision as abortive hatching may lead to egg depletion (Brendonck, 1996). Although there are many suggestions how to enhance the hatching success of invertebrate resting eggs (Brendonck, 1996), its drivers are still not fully understood, and results may vary considerably between populations and within populations of the same species. Moreover, differences seem to exist even between eggs from the same brood (Hildrew, 1985; Brendonck, 1996; Simovich and Hathaway, 1997; Pinceel *et al.*, 2013; Wang and Chou, 2015).

CONCLUSIONS

We found that osmotic pressure caused by sugar solution, centrifugation and subsequent placement in much more diluted medium (as part of the Onbé-Marcus sugar flotation method) cannot be considered as a hatching trig-

ger for branchiopod eggs, and neither can it cause any significant changes in the hatching success of the examined branchiopod species. Our results showed that the contribution of the sugar flotation to a higher number of hatchlings and faster emergence (Vandekerckhove *et al.*, 2004) is due to the effect of removing the sediment covering the eggs, and compared to hand-picked isolation, it does not increase hatching rates or speed. On the other hand, our results also prove sugar flotation as a non-invasive method so it can be used also when the number of eggs in a sample is limited. Therefore, application of the method is advisable for studying branchiopod species, since it facilitates egg tracing within the sediment and contributes to the exposition of eggs to light, which seem to be its major advantages.

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