Pesticide exposure impacts not only hatching of dormant eggs, but also hatchling survival and performance in the water flea *Daphnia magna*

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Abstract Laboratory ecotoxicity tests and biomonitoring in aquatic systems are currently based on the active component of invertebrate communities. Even though dormant egg banks are crucial for the long term survival and community dynamics of many aquatic organisms, the effects of anthropogenic activities on dormant egg bank dynamics have rarely been studied. In this study we investigated the effects of two pesticides with a different mode of action (carbaryl and fenoxycarb) on hatching of *Daphnia magna* dormant eggs (ephippia) as well as on survival, growth and reproduction of the hatched neonates. Dormant eggs were exposed to the pesticides simultaneously to incubation under conditions that induce hatching (long daylight and 20 °C). Carbaryl had no negative effects on embryonic development or hatching rate up to concentrations almost 1,000 times the median effect concentration (EC₅₀) of neonate survival in acute tests. Fenoxycarb, however, had a significant dose-related effect by delaying or completely stopping the hatching process and caused severe abnormalities in developing individuals. Both pesticides had significant negative effects on survival and reproduction of the hatchlings. These results indicate that, in addition to inducing mortality of active individuals, pesticides can affect zooplankton communities by altering hatching dynamics and life history traits of hatched individuals. We briefly discuss how such pollution induced changes in the benthic–pelagic coupling could translate into trans-

generational effects impacting ecological and evolutionary dynamics.

Keywords Dormant egg banks · Ehippia · Embryonic development · *Daphnia magna* · Carbaryl · Fenoxycarb

Introduction

Many invertebrate species living in permanent and temporary standing waters produce dormant stages to survive unfavourable environmental conditions, such as drought, low oxygen concentrations, food limitation, crowding and the presence of predators (Hairston et al. 1990; Alekseev and Starobogatov 1996; Brendonck et al. 1998; Slusarczyk 1999, Slusarczyk et al. 2005). Dormant eggs accumulate over the years in the sediment to form a dormant egg bank, analogous to plant seed banks (De Stasio 1989; Hairston and Cáceres 1996), with each growing season only a fraction of the dormant eggs hatching. Through this benthic–pelagic coupling, events in the dormant phase can affect the active, aquatic phase and vice versa (Cáceres and Hairston 1998; Gyllström and Hansson 2004). Zooplankton dormant eggs can remain viable for hundreds of years (Hairston et al. 1995; Cáceres 1998). With densities in the sediment ranging between 10³ and 10⁶ eggs/m² (Hairston 1996; Brendonck and De Meester 2003; Vandekerckhove et al. 2005a), the dormant fraction is far from negligible and may strongly impact ecological and evolutionary dynamics (Gyllström and Hansson 2004). When environmental conditions fluctuate, egg banks can function as a reservoir of species and genetic diversity through a mechanism called the “storage effect” (Chesson and Warner 1981; Cáceres 1997). Dormant egg banks integrate genetic variation that has accumulated over several growing seasons

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(Ellner and Hairston 1994; De Meester et al. 2006) thereby increasing the evolutionary potential of a population (Hairston and De Stasio 1988; Hedrick 1995; Brendonck and De Meester 2003). Despite its importance in ecological and evolutionary processes (reviewed in Brendonck and De Meester 2003), dormant egg bank dynamics are rarely included in zooplankton population and community studies (Cáceres 1998; Hairston et al. 2000) and there is almost no information available on the effects of pollution on dormant egg bank dynamics.

Daphnia magna, a well established model organism and standard test species in ecotoxicology (Walker et al. 2001), reproduces by cyclical parthenogenesis. Production of sexual, dormant encapsulated eggs (ephippia) is triggered by conditions announcing or associated with an unfavorable environment (Hebert 1978; Decaestecker et al. 2009). Standard ecotoxicity tests with *Daphnia* (OECD 2004, 2012) generally focus on the effects of chemicals on the asexual part of the reproduction cycle, screening clonal lineages. However, chemical compounds can interfere with the life cycle of *Daphnia* through a number of different pathways: they can (i) alter the hatching rate of dormant eggs, either by impacting the embryos (Fig. 1, number 1) or by changing the response to hatching cues (Fig. 1, number 2) (Angeler et al. 2005; Raikow et al. 2006, 2007); (ii) affect survival and growth of hatched individuals (Fig. 1, number 3); (iii) change the fitness and reproductive traits of parthenogenetic females (Fig. 1, number 4) (Barata et al. 2007; Hassold and Backhaus 2009; Jansen et al. 2011); (iv) change offspring sex ratio (Fig. 1, number 5) (Dodson et al. 1999; Olmstead and LeBlanc 2003; Tatarazako and Oda 2007; Palma et al. 2009); and (v) affect timing and amount of dormant egg production (Fig. 1, number 6) (Shurin and Dodson 1997; Olmstead and LeBlanc 2001; Ignace et al. 2011).

Little information is available on the effects of chemical exposure on survival and hatching of zooplankton dormant eggs in general, and in *Daphnia* more specifically. A few studies evaluated the efficiency of ship ballast tank treatments, in order to avoid introduction of exotic/invasive species through their dormant stages. The biocides sodium hypochloride and menadione (SeaKleen) affected hatching success of *D. mendotae* dormant eggs, but effect levels were higher compared to other *D. mendotae* life stages (Raikow et al. 2006, 2007). Angeler et al. (2005) tested for effects of fire retardant treatments and found a significant negative effect of Fire Trol 943 on emergence of *D. curvirostris* dormant eggs from wetland sediments. For rotifers, Marcial et al. (2005) found that diazinon, fenitrothion, methoprene and isoprothiolane affected hatching of *Brachionus plicatilis* dormant eggs at concentrations 2–40 times lower than concentrations affecting population growth, mixis and fertilization endpoints. Marcial and

Hagiwara (2007) discovered that *B. plicatilis* hatching rates were severely affected by diazinon, but only when rotifers were exposed during dormant egg production or shortly afterwards. Copepod dormant eggs (*Acartia pacifica*) were much more sensitive to metals than benthic adults (Jiang et al. 2007). The results of different studies using *Artemia* cysts, however, appear to be inconclusive. Varó et al. (2006) and Sarabia et al. (2003, 2008) reported no adverse effects of metal and pesticide exposure on hatching of *Artemia* cysts. Bagshaw et al. (1986) and Rafiee et al. (1986), on the contrary, reported that hatching of dormant eggs was more sensitive to heavy metal exposure than hatched individuals.

In this study we have tested for acute and chronic effects of pesticide exposure on dormant eggs of *D. magna*. Therefore we have conducted two series of experiments: in a first series we focused on the effects of pesticides on embryonic development and hatching characteristics of the dormant eggs (Fig. 1, number 1+2), in a second experiment we tested whether exposure to pesticides could have long term effects on survival and life history characteristics of the hatched neonates (Fig. 1, number 3+4). For our experiments, we selected two model pesticides with a different mode of action: carbaryl and fenoxycarb. Carbaryl is a carbamate insecticide, which causes overstimulation of the nervous system by binding to acetylcholine esterase (Walker et al. 2001), and has a very high acute toxicity for crustaceans (EFSA 2006). Fenoxycarb, on the other hand, is an insect growth regulator, which is known to disturb the endocrine system in crustaceans (LeBlanc 2007), by changing offspring sex ratio (Tatarazako and Oda 2007) and causing developmental malformations in parthenogenetic eggs (Mu and LeBlanc 2004). Our working hypothesis is that exposure to pesticides, if it coincides with incubation under optimal hatching conditions, might impact embryonic development and hatching rate of the dormant eggs, as well as life history characteristics of the hatched neonates. We expect the effects to be different for the two model pesticides, with a more pronounced effect of fenoxycarb, known to affect developmental processes in invertebrates.

Materials and methods

Daphnia magna ephippia

Daphnia magna ephippia from two different sources were used; (1) from a field population ‘Langerodevijver’ (LRV: Korbeek-Dijle, Belgium) and (2) from a standardized laboratory culture (MBT: MicroBioTests Inc., Mariakerke, Belgium). The LRV field population was selected because it is located in a rather pristine environment (natural

Fig. 1 Toxicants can have an effect on different stages of the *Daphnia* reproduction cycle: (1) direct mortality of the dormant eggs; (2) effects on hatching characteristics of the dormant eggs; (3) effects on growth; (4) effects on asexual reproduction; (5) effects on sexual

reproduction (through changes in sex ratio) and (6) effects on dormant egg production. This study is focused on pathways 1, 2, 3 and 4, following toxicant exposure of the dormant eggs

reserve 'Doode Bemde': Orsini et al. 2012) and has a high density of *D. magna* ehippia (Rousseaux 2011). The dormant egg bank was sampled in the winter period (December 2011) since the dormant egg bank then has its maximum size and eggs are in diapause. A plexiglass sediment corer (diameter 5 cm, length 1 m) was used for sampling at 10 locations in the open water zone of the lake. Only the top 5–10 cm of the cores (active egg bank: Cáceres and Hairston 1998) were retained. Pooled sediment samples were sieved over 1 mm and 250 µm sieves and *D. magna* ehippia were isolated and stored for at least 1 month in the dark at 4 °C (according to Vandekerckhove et al. 2005b). MBT ehippia are internationally used in standard acute toxicity assays and hatched neonates have a sensitivity similar to that of parthenogenetic offspring from lab cultures (Persoone et al. 2009). All ehippia were mechanically decapsulated with metal tweezers shortly before the start of the experiments and only healthy eggs were used.

Effects on embryonic development and hatching characteristics

In a first range-finding experiment a wide range of carbaryl and fenoxycarb concentrations were applied to find effect

levels on hatching characteristics of LRV dormant eggs. Carbaryl (1-naphthyl methylcarbamate, CAS no. 63-25-2, 99.8 % purity, Sigma-Aldrich, Germany) and fenoxycarb (ethyl 2-(4-phenoxyphenoxy)ethylcarbamate, CAS no. 72490-01-8, 99.6 % purity, Sigma-Aldrich, Germany) were dissolved in absolute ethanol (purity min. 99.8 %, VWR International, France). The concentration of ethanol was the same in all treatment solutions and in the solvent control (0.005 % ethanol). Exposure solutions were freshly prepared with final concentrations of 5, 50, 500, 1,000 and 5,000 µg/L for carbaryl and 0.5, 5, 50, 500, and 5,000 µg/L for fenoxycarb. These concentrations were chosen based on the sensitivity of *Daphnia* neonates as established in standard acute ecotoxicological tests (EFSA 2006; Coors et al. 2009; EFSA 2010) and previous preliminary experiments (S. Navis, unpublished data). For carbaryl we choose concentrations up to 1,000 times the EC₅₀ (6–17 µg/L) for neonate immobilisation. For fenoxycarb we choose a range of concentrations from 1,000 times below the EC₅₀ for neonate immobilisation (500–600 µg/L) up to 10 times above, because we expected effects on embryonic development at low concentrations from earlier work by Mu and LeBlanc (2004).

Although no longer registered for use in Europe, carbaryl is still widely used in amongst others, the United

States, Canada and Australia. Observed field concentrations for carbaryl are generally low, ranging from below the detection limit up to 5.5 µg/L (US Environmental Protection Agency 2003). However, peak concentrations of 1.74 and 4.8 mg/L have been reported in periods immediately following application (Norris et al. 1983; Bridges 1999; Walters et al. 2003). Tested carbaryl concentrations are thus in the range of measured environmental concentrations. For fenoxycarb no field measurements of small water bodies were found in literature. Environmental concentrations (PEC) in surface waters immediately after application of 87.6 µg/L have been predicted (FOCUS 1 scenario; EFSA 2010).

Dormant eggs were placed individually in the wells of a 24-well microtiter plate (polystyrene, non coated, sterile plates, Greiner Bio-One GmbH) containing 2 mL of exposure medium, prepared in artificial freshwater (ADaM: Klüttgen et al. 1994). Per treatment 48 dormant eggs (replicates) were used. Treatments were randomly allocated to plates in such a way that each treatment was allocated to four randomly assigned half multiwell plates. The influence of plate identity was taken into account by including it as a random blocking factor in the statistical analyses. Plates were incubated for 10 days at 20 ± 2 °C in a light:dark regime of 18:6 h, to simulate spring conditions. Dormant eggs were checked daily for hatching.

In a second experiment, a more narrow range of fenoxycarb concentrations (50, 125, 250, 500, 1,000, 2,000 and 4,000 µg/L) was selected, based on the results of the range-finding experiment, to test for effects on both embryonic development and hatching characteristics of the dormant eggs. This was not done for carbaryl, since even the highest test concentration in the range-finding experiment did not have a significant effect. Test procedures were identical to the previous experiment; plates were incubated for 10 days and dormant eggs were checked daily for hatching. In addition, during day 3–7 of the experiment, embryonic development and morphological abnormalities were monitored using a digital camera (Olympus Colour view III) attached to an Olympus CKX41 inverted microscope (magnification 10×).

For this series of experiments, developmental abnormalities of the embryos as well as hatching rate (cumulative hatching %) related to pesticide exposure were statistically evaluated using generalized linear models (GLM) with a logit-link function and binomial distribution, followed by sequential Bonferroni-correction (Holm 1979). Effects of the pesticides on timing of hatching (day of maximum hatching) were evaluated for the two pesticides separately using one-way ANOVA's followed by Tukey's HSD post hoc tests. Concentration–response curves and effect levels (EC₅₀ values) were estimated using the DRC-package in R (Ritz and Streibig 2005).

Life cycle toxicity: from hatching to reproduction

In a second series of experiments, the effects of carbaryl and fenoxycarb exposure, on growth, maturation and reproductive capacity of *D. magna* were assessed by adapting standard methods for chronic toxicity tests (OECD 2012). Based on results of the first series of experiments, three test concentrations for each pesticide were selected: 50, 500 and 5,000 µg/L for carbaryl and 500, 750 and 1,000 µg/L for fenoxycarb. Dormant eggs from both LRV and MBT were used in a full factorial design: 2 populations × 2 pesticides × 4 pesticide concentrations (incl. control) = 16 treatments. Two controls were included: a blank control containing only ADaM-water and a solvent control with 0.005 % ethanol (equal to the ethanol concentration in all exposure solutions). Dormant eggs were individually placed in 24-well microtiter plates, with a total of 48 eggs (replicates) per treatment and each treatment was randomly divided over four half plates. To ensure that exposure to the pesticides could only occur during the hatching process and not after the organisms had hatched, the eggs were transferred into new microtiter plates with 2 mL fresh ADaM-water at the end of the second day of exposure (before any eggs had hatched) and rinsed once more to ensure that no exposure medium could be present in the wells. Hatching was checked daily and from each treatment 15 randomly selected hatchlings (replicates) from the day of maximum hatching were transferred individually into 100 mL glass beakers with ADaM-water. Daphnids were fed daily with *Scenedesmus obliquus* (1×10^5 cells/mL), and medium was renewed every 2 days. All individuals were monitored daily for at least 21 days, or until release of the second brood. Offspring were counted and removed from the test vessels daily. Survival, age at maturation, age at release of the first and second clutch and clutch size of first and second brood were recorded. The performance (*r*) was computed iteratively from the Euler–Lotka equation (Begon et al. 2005) and provides an estimate of the intrinsic rate of increase under the assumption of the absence of mortality:

$$\sum_{x=0}^{\infty} e^{-rx} l_x m_x = 1$$

where l_x represents the proportion of individuals surviving to age x (days; here we calculated performance only for surviving individuals so l_x is assumed to be 1) and m_x is the number of juveniles produced by the individual between the ages x and $x + 1$. Performance was calculated only for the first brood, since some individuals died before producing a second brood. To monitor growth, body size of all experimental organisms was monitored by photographing each individual directly after hatching, after 10 and 21 days as well as at maturation and at time of first and

second reproduction. In addition, from each individual three neonates per brood were photographed. All images were recorded using a digital camera (Olympus Colour view III) attached to an Olympus BX50 microscope (magnification adults 2×, neonates 4×) and processed later for measurements of body length (from top of the eye to base of the tail spine).

Main effect of pesticide and population (origin of the dormant eggs) and the pesticide × population interaction effect on survival of the hatched neonates, were tested for each pesticide separately using GLM with a logit-link function and binomial distribution, followed by a sequential Bonferroni-correction. Effects of pesticide and population origin on life history traits (growth and reproduction) were assessed for the two pesticides separately using two-way ANOVA's followed by Tukey's HSD post hoc tests.

All statistical analysis were performed in R statistical software v2.15.0 (R Development Core Team 2012). All pesticide concentrations mentioned are initial nominal concentrations. In all experiments a blank control and a solvent control treatment were included. If the results of both control treatments were not statistically different, only results from the blank controls were reported.

Results

Carbaryl

Exposure of LRV dormant eggs to carbaryl concentrations up to 5,000 µg/L had no significant effects on hatching characteristics. Cumulative hatching percentages after 10 days ranged from 52.1 to 81.3 %, but there was no concentration–response relationship ($\text{Chi}^2 = 10.12$; $\text{df} = 5$; $p = 0.072$). The NOEC for hatching in the presence of carbaryl is therefore $\geq 5,000$ µg/L.

Carbaryl did have a significant negative impact on survival of the hatched individuals (Fig. 3a, c). For MBT dormant eggs, survival in the control treatment was 86.7 % and all three tested carbaryl concentrations resulted in significantly reduced survival compared to the control (Fig. 3a). For LRV dormant eggs, survival in the control treatment was 53.3 % and only the highest tested carbaryl concentration of 5,000 µg/L had a significant negative impact on survival (Fig. 3c).

Carbaryl also significantly affected other life history traits (Table 1). For MBT, all three tested carbaryl concentrations caused a significant reduction in performance (r), a higher age at maturity and at release of the first and second brood, a lower clutch size (first and second brood) and a smaller body size (measured after 21 days, corrected for body size directly after hatching) (Fig. 4). No significant effects of carbaryl on offspring body size of the first

brood were detected, but offspring of the second brood was significantly smaller in carbaryl treatments compared to the controls. For LRV, only significant negative effects were found for age at first reproduction at the highest test concentration. Other measured traits did not differ significantly from the controls. The LRV population showed very large variation in responses within treatments, also for the controls, especially regarding clutch sizes. In addition, survival at the highest pesticide concentrations was very low ($n = 3$), resulting in reduced statistical power.

Fenoxycarb

Fenoxycarb had a significant negative effect on hatching of *D. magna* dormant eggs (LRV). In the preliminary range-finding experiment, hatching at the highest tested fenoxycarb concentration (5,000 µg/L) was completely inhibited ($\text{Chi}^2 = 79.47$; $\text{df} = 5$; $p < 0.001$). In the final experiment, hatching rate was significantly negatively impacted at concentrations 2,000 and 4,000 µg/L (cumulative hatching was respectively 6.3 and 0 %; $\text{Chi}^2 = 267.78$; $\text{df} = 7$; $p < 0.001$) (Fig. 2a) and an EC_{50} for hatching of 1,300 µg/L was estimated. Timing of hatching was delayed at concentrations above 250 µg/L ($F = 56.28$; $\text{df} = 6$; $p < 0.001$). In the control treatment, the peak of hatching occurred at day 3, while in the fenoxycarb treatments maximum hatching occurred at day 4–7. Analysis of the photographs of the developing embryos revealed that fenoxycarb caused developmental abnormalities (Fig. 2b), including poorly developed second antennae, morphological deformations of the tail spine, carapace and compound eye. Embryonic development was severely altered or arrested at concentrations of 1,000, 2,000 and 4,000 µg/L fenoxycarb, with abnormalities observed in respectively 79.2, 85.4 and 93.8 % of the embryos. At 1,000 µg/L, 56.3 out of 79.2 % abnormally developed embryos still hatched. At higher test concentrations almost none of the deformed embryos was able to hatch (2.1 and 0 % at respectively 2,000 and 4,000 µg/L).

Fenoxycarb had a significant negative impact on survival of the hatched individuals. For MBT, all three tested concentrations resulted in significantly reduced survival compared to the control treatments (Fig. 3b). For LRV dormant eggs, only the highest tested concentration of 1,000 µg/L had a significant negative impact on survival (Fig. 3d).

Fenoxycarb also had a significant negative effect on life history traits of the surviving individuals (Table 1). For MBT, all three test concentrations caused a decrease in performance (r), a higher age at maturity and at release of the first and second brood and a lower clutch size (first and second brood) (Fig. 4). For the highest concentration

Fig. 2 Effects of fenoxycarb treatments on **a** cumulative hatching percentage during 10 days after exposure and **b** the percentage of deformed hatchlings ($n = 48$). Fenoxycarb both decreased the hatching success and increased the percentage of deformations in a dose-dependent manner. Distinct letters in the figures indicate

significant differences among treatments ($p \leq 0.05$, generalized linear model, followed by sequential Bonferroni-correction). The photographs represent (from top to bottom): a normal developing dormant egg from the control treatment; an embryo with developmental malformations; and a deformed hatchling

Fig. 3 Cumulative survival percentage of hatched individuals from different pesticide treatments ($n = 15$, replicates) during the 21 days of the experiment: **a** carbaryl, hatched from dormant eggs produced in the laboratory under controlled conditions (MBT); **b** fenoxycarb, hatched from MBT; **c** carbaryl, hatched from a natural source of

dormant eggs (LRV); **d** fenoxycarb, hatched from LRV. Distinct letters in the figures indicate significant differences among treatments ($p \leq 0.05$, generalized linear model, followed by sequential Bonferroni-correction)

there was also a negative effect on growth of the experimental organisms. Again no significant effects on offspring body size (first and second brood) were detected. For LRV, all three fenoxycarb concentrations had

significant negative effects on performance and fenoxycarb 750 µg/L had a significant negative effect on age at first brood, but no significant effects on other life history traits were detected.

Table 1 Results of the 2-way ANOVA on the life history traits of *D. magna* for the two model pesticides

	Concentration (df = 3)		Population (df = 1)		Conc*population (df = 3)	
	F	P	F	P	F	P
Carbaryl						
Performance (r)	10.34	<0.001	18.75	<0.001	15.40	<0.001
Age at maturity	11.65	<0.001	34.61	<0.001	9.63	<0.001
Age at 1st brood	12.22	<0.001	37.44	<0.001	18.88	<0.001
Age at 2nd brood	5.42	0.003	24.95	<0.001	11.94	<0.001
Size of 1st brood	6.34	<0.001	5.62	0.022	7.58	<0.001
Size of 2nd brood	2.43	0.077	8.16	0.006	4.65	0.006
Body size at hatching	4.69	0.004	3.49	0.064	0.78	0.509
Growth hatching—day 11	24.13	<0.001	59.08	<0.001	16.97	<0.001
Growth hatching—day 21	4.30	0.009	11.93	0.001	4.67	0.006
Body size of offspring 1st brood	0.10	0.960	0.09	0.759	0.84	0.477
Body size of offspring 2nd brood	5.88	0.002	12.60	<0.001	3.41	0.025
Fenoxycarb						
Performance (r)	30.53	<0.001	4.25	0.045	0.49	0.691
Age at maturity	16.24	<0.001	5.59	0.022	3.35	0.026
Age at 1st brood	17.20	<0.001	6.53	0.014	2.94	0.042
Age at 2nd brood	13.59	<0.001	0.82	0.371	1.43	0.251
Size of 1st brood	20.43	<0.001	1.46	0.234	0.44	0.723
Size of 2nd brood	5.38	0.003	0.40	0.529	0.17	0.844
Body size at hatching	8.42	<0.001	31.87	<0.001	0.96	0.414
Growth hatching—day 11	24.36	<0.001	2.80	0.099	0.60	0.617
Growth hatching—day 21	5.93	0.002	3.41	0.071	1.14	0.343
Body size of offspring 1st brood	0.32	0.813	0.01	0.923	1.15	0.341
Body size of offspring 2nd brood	4.25	0.010	2.84	0.099	0.50	0.610

Discussion

Our results reveal the effects of two model pesticides with a different mode of action on hatching characteristics of *D. magna* dormant eggs and on subsequent survival and life history characteristics of hatched individuals. Overall, our results indicate that dormant eggs are quite insensitive to carbaryl with respect to hatching rates, while they are influenced by fenoxycarb at relatively moderate concentrations. Survival and performance of hatchlings were impacted by both pesticides. We discuss these results and highlight the possible impact of the observed effects on the development and dynamics of zooplankton populations in natural systems.

Impact of pesticides on hatching, growth and reproduction

Carbaryl did not have a significant negative impact on hatching up to the highest tested concentration

(NOEC $\geq 5,000$ $\mu\text{g/L}$). This is a striking observation as acute effect levels of neonate immobilisation reported for carbaryl range between 6 and 17 $\mu\text{g/L}$ (EFSA 2006; Coors et al. 2009), i.e. 300 to almost 1,000 times lower concentrations than used in our experiment. This indicates that for carbaryl, hatching and development of *D. magna* dormant eggs is a much less sensitive endpoint than neonate immobilisation, as used in standard acute toxicity tests. For fenoxycarb, however, we observed clear dose-related effects on dormant eggs, resulting in a delay in hatching or even complete absence of hatching. The EC₅₀ of fenoxycarb for hatching is 1,300 $\mu\text{g/L}$, which is about twice as high as the acute effect level for neonate immobilisation of 500–600 $\mu\text{g/L}$ (EFSA 2010). Fenoxycarb also caused severe abnormalities in developing individuals at 1,000 $\mu\text{g/L}$ or higher test concentrations, which is again above the EC₅₀ for neonate immobilisation. Fenoxycarb is known to cause similar malformations in parthenogenetic eggs, but at much lower (1,000 times) concentrations than found in our study (Mu and LeBlanc 2004). Overall, we can conclude

Fig. 4 Effects of carbaryl (carb) and fenoxycarb (fnx) exposure on growth and reproduction (average $\pm$ 1 SE) of *D. magna* hatchlings (number of replicates (n) depends on survival of the hatched individuals, in panels A–F from left to right and per population; MBT control: 15, carb50: 5, carb500: 5, carb5000: 2, and fnx500: 11, fnx750: 4, fnx1000: 5, LRV control: 9, carb50: 10, carb500: 9, carb5000: 3, fnx500: 5, fnx750: 6, fnx1000: 2, and in panels G–H; MBT control: 13, carb50: 4, carb500: 7, carb5000: 3, and fnx500: 10, fnx750: 4, fnx1000: 6 and for LRV control: 8, carb50: 10, carb500: 9, carb5000: 3, fnx500: 5, fnx750: 7, fnx1000: 1). Letters above each result indicate significant differences among treatments ($p \leq 0.05$, 2-way ANOVA, Tukey's HSD post hoc test; capital letters are used for the MBT population and lower case for the LRV population)

that, for both model pesticides, *D. magna* was less sensitive when pesticide exposure occurs during embryonic development and hatching than during other life stages. A possible explanation for the lower sensitivity at this stage may be that dormant eggs are better protected against penetration of pesticides. Besides the protective envelope, which was removed in our experiments through decapsulation, they have thick, multi-layered egg membranes which enable them to endure extreme physical conditions like freezing, desiccation and bird digestion enzymes (Mellors 1975; Frisch et al. 2007). Varó et al. (2006) demonstrated that the chorion surrounding cysts of *Artemia* can act as a

barrier retaining the majority of the toxicant. However, our results do indicate that the pesticides were able to enter the dormant eggs, as both pesticides had significant negative effects on hatchling survival, growth and reproduction, even though the eggs were only exposed during the first 2 days of the hatching process and were transferred to clean medium before hatching occurred. Our observation that effects were only observed at relatively high concentrations, corresponding to environmental concentrations measured shortly after application, may reflect that the concentration of the pesticides inside the eggs was relatively low compared to the nominal concentrations used in

the exposure medium. This might also explain why carbaryl had no significant effects on hatching characteristics up to the highest concentration tested, but did have significant chronic effects, that typically occur at lower exposure levels. Moreover, this study is the first to demonstrate that exposure to toxicants in the dormant phase cannot only affect hatching rates, but also results in reduced reproductive rates of hatched individuals.

The large differences between carbaryl and fenoxycarb regarding their effects on hatching and life history characteristics of *D. magna*, can at least partly be explained by their different mode of action. Carbaryl is a carbamate insecticide with neurotoxic activity, causing overstimulation of the nervous system (Walker et al. 2001). Fenoxycarb, on the other hand, is a juvenile hormone analogue and a very potent methyl farnesoate agonist (Tatarazako et al. 2003; Oda et al. 2005; Wang et al. 2005) that is able to induce the production of male neonates in *D. magna* at concentrations as low as 100 ng/L (Tatarazako and Oda 2007). Fenoxycarb can also disrupt ecdysteroid-regulated aspects of embryo development in parthenogenetic eggs, but at levels 10 times higher compared to effects on offspring sex ratio (Mu and LeBlanc 2004). Due to their different mode of action, fenoxycarb was expected to interfere with embryonic development while carbaryl was expected to have an effect mainly when the nervous system has developed. This was largely confirmed in our study.

Despite the importance of dormant egg banks for the persistence of zooplankton communities in general and *Daphnia* populations in particular (Brendonck and De Meester 2003), little information is available on the effects of chemical exposure on survival and hatching of dormant eggs. Significant negative effects of toxicants on hatching of *Daphnia* dormant eggs have been reported previously in a few studies (Angeler et al. 2005; Raikow et al. 2006, 2007). Our findings are in agreement with the results of Raikow et al. (2006, 2007), who observed that the biocide SeaKlean had significant negative effects on hatching of *D. mendotae* dormant eggs, but with a ten times lower sensitivity compared to neonate mortality (Song et al. 2011). Studies using dormant eggs of other zooplankton species (mainly rotifers and *Artemia*) gave variable results; ranging from no significant adverse effects on hatching rates (Sarabia et al. 2003, 2008; Varó et al. 2006; Marcial and Hagiwara 2007), to effects on unhatched embryos at concentrations below effect levels for hatched individuals (Bagshaw et al. 1986; Rafiee et al. 1986). This indicates that effects of chemicals on dormant stages differ among and within species as well as among toxicants, depending on their mode of action.

In our life cycle toxicity experiment we have used dormant eggs from two different populations; one laboratory population (MBT) and one field population (LRV).

Control survival of the MBT clones was higher than of the LRV clones, which might be explained by the fact that MBT ephippia have been produced under similar laboratory conditions as used in our experiments (Persoone et al. 2009). Also, MBT clones appeared more sensitive to pesticide exposure than the ones from LRV. Genetic variability in response to stressors has been frequently observed, both among and within populations (Barata et al. 2002; Oda et al. 2006, 2007). Oda et al. (2007), for example, reported a variation in estimated EC_{50} values for fenoxycarb by a factor of four, between seven strains of *D. magna* from different laboratories. Since dormant eggs are produced by sexual reproduction, each individual hatchling represents a different clone and may reveal somewhat different characteristics and tolerances.

Ecological implications

In this study we have shown that pesticides can have negative effects on hatching and development of *D. magna* dormant eggs under conditions where exposure was maximized: the protective envelope was removed and the dormant eggs were exposed to both optimal hatching conditions and pesticides simultaneously. This scenario might be comparable to pesticide applications during spring, resulting in high concentrations of pesticides in small water bodies (via spray drift, runoff or drainage) coinciding with a peak in hatching from the dormant egg bank. The potential impact of the pesticides on the dormant fraction of the community does not only depend on the application time, but also on the exposure route of the respective pesticides. Fenoxycarb is known to dissipate from the water phase quite rapidly and then to bind to the organic phase of the sediment (half-life in water = 3.9 days, half-life in water-sediment systems = 18.8 days; Sullivan 2000). This indicates that sediment might be an important exposure route. This was confirmed by Licht et al. (2004) and Jungmann et al. (2009) who reported negative effects of sediment spiked with fenoxycarb on, respectively, mayfly metamorphosis and emergence of chironomids. It is therefore important that future research on dormant stages also focuses on the effects of chemicals under different environmentally realistic scenarios in order to get more insight into the importance of the exposure route (water vs. sediment exposure), timing of exposure and the importance of the ephippial case in protecting dormant eggs from exposure to pollutants.

There are different pathways in which pollution can affect structure and functioning of aquatic communities, or interfere with the life cycle of *D. magna* (Fig. 1). Our observation that even a brief exposure to pesticides during the early stages of embryonic development of dormant eggs leads to reduced hatching rates (fenoxycarb; pathway

2 Fig. 1), a higher proportion of deformed embryos (fenoxycarb; pathway 1+2 Fig. 1) and higher mortality and decreased performance of hatchlings (both pesticides; pathway 3+4 Fig. 1) can have far-reaching consequences for ecological and evolutionary dynamics of zooplankton in lakes and ponds. Reduced hatching rates and performance will reduce population growth rates. A reduced build-up of *Daphnia* early in the season may impact algal growth (reduced top-down control of algae) and fish (less food), and to the extent that the responses are species specific, they may impact species composition of the zooplankton communities. Arbačiauskas and Gasiunaite (1996) and Arbačiauskas and Lampert (2003) showed that ex-ephippial individuals are characterized by different life history traits, fostering faster population growth rates. The impact of pesticides on hatching rates and the fitness of hatchlings thus may even have a higher impact given the importance of the ex-ephippial generation to population development. In addition, repeated pesticide exposure, leading to reduced hatching rates and mortality, may eventually gradually exhaust the dormant egg bank. Since dormant egg banks integrate genetic variation that has accumulated over many growing seasons (Ellner and Hairston 1994; De Meester et al. 2006), a decrease in size of the dormant egg bank or a reduced contribution of the dormant to the active phase may reduce genetic variation, hence the evolutionary potential of a population (Brendonck and De Meester 2003). This makes exposed populations more vulnerable to changes in environmental conditions of both natural and anthropogenic origin. Despite the importance of dormant egg banks for ecological and evolutionary processes, experimental evidence regarding the influence of toxicants on dormant egg bank dynamics is surprisingly scarce. A better understanding of the effects of toxicant exposure on the full life-cycle of zooplankton species, especially of the important model organism *D. magna*, could increase the ecological relevance of ecotoxicity testing and aid our understanding of not only the impact of pesticide exposure, but also the potential for recovery in aquatic ecosystems.

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