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Chronic Effects of Carbamazepine on Life-History Strategies of *Ceriodaphnia dubia* in Three Successive Generations

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Abstract Trace quantities of pharmaceuticals are continuously being discharged into the environment through domestic and industrial wastewater effluents, causing concern among scientists and regulators regarding potential long-term impacts on aquatic ecosystems. These compounds and their metabolites are constantly interacting with organisms at various life-cycle stages and may differentially influence the development of embryonic, larval, juvenile, and adult stages. To understand the possible cumulative effects of exposure to carbamazepine (CBZ), a multigenerational approach was taken in which survival, reproduction, respiration, growth, brood size, and biomass of *Ceriodaphnia dubia* were assessed at sublethal concentrations over the course of three successive generations. CBZ exposure significantly decreased fecundity at 196.7 μg/L in the F0 and F1 generations over 2 weeks and acclimatized at 264.6 μg/L in the F2 generation. Similarly, a significant decrease of neonate dry weight was observed at the 196.7 μg/L CBZ treatment in the F1 generation, and it acclimatized at 264.6 μg/L treatment level in the F2 generation. Median time to first brood release was significantly delayed at 264.6 μg/L in the F2 generation, indicating slower maturation. Results over three successive generations are not different than what one would obtain by testing simply the F0 generation. Furthermore, the effects measured were observed at concentrations two orders of magnitude higher than are environmentally relevant, and it is unlikely that CBZ poses a substantial risk to the environment regarding the end points measured in this study. However, additional research through laboratory and field multigenerational studies may be required to understand the overall risk of CBZ to other nontarget organisms.

Pharmaceuticals are continuously discharged into the environment through domestic and industrial wastewater effluents, resulting in the chronic exposure of aquatic organisms to these compounds in surface waters and wastewaters worldwide in the ng/L to μg/L range (Kolpin et al. 2002; Focazio et al. 2008; Constantine and Huggett 2010). Pharmaceuticals can affect aquatic organisms at sublethal concentrations by altering the physiological processes of individual organisms (Muller et al. 2010; Gust et al. 2011). Carbamazepine (CBZ), a commonly prescribed antiepileptic drug, has been detected in rivers, streams, and wastewaters worldwide at levels ranging from 325 ng/L to 6.3 μg/L with a reported detection frequency of >90 % in wastewater effluents throughout the United States (Ferrari et al. 2004; Kolpin et al. 2004; Glassmeyer et al. 2005; Fent et al. 2006; Leclercq et al. 2009; Zhang and Geißen 2010). Its pseudo-persistent nature in the environment is likely due to its low removal efficiency by

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wastewater treatment plants, calculated to be <7 %, and its relatively long half-life of 37.5 days (Daughton and Ternes 1999; Andreozzi et al. 2002; Ferrari et al. 2003, 2004; Metcalfe et al. 2003, 2004; Tixier et al. 2003; Nentwig et al. 2004; Brun et al. 2006; Ying et al. 2009; Meredith-Williams et al. 2012). The ratio between the predicted environmental concentration in the aquatic environment and the predicted no-effect concentration in effluents in Germany was 47 (Ferrari et al. 2003, 2004), indicating that CBZ is potentially a hazardous chemical. Furthermore, CBZ has been reported at environmental concentrations that can induce effects (Contardo-Jara et al. 2011).

CBZ is neuroactive and interacts with receptors in target and nontarget organisms. The main mode of action of CBZ in mammals is the stabilization of inactivated states of sodium channels, whereby neurons become less excitable and decrease action potential (Contardo-Jara et al. 2011). Furthermore, one of its major metabolites, CBZ-10,11-epoxide, is known to have similar antiepileptic properties as CBZ (Miao and Metcalfe 2003; Miao et al. 2005) and could pose an ecotoxicological risk (la Farré et al. 2008). Although the exact mode of action is unknown in invertebrates, it is conceivable that CBZ and active metabolites may interact with invertebrate Na^+ channel. The most frequently reported effects of CBZ include oxidative stress and protein damage in *Dreissena polymorpha* (Contardo-Jara et al. 2011), growth retardation in algae and cladocerans (Jos et al. 2003; Cleuvers 2003, 2004; van den Brandhof and Montforts 2010; Dietrich et al. 2010; Zhang et al. 2012), developmental abnormalities in *Danio rerio* (Crane et al. 2006), reproductive impairment in benthic insects *Chironomus riparius* and *Hyalella azteca* (Nentwig et al. 2004; Oetken et al. 2005; Dussault et al. 2008a, b), behavior modification in *Dugesia tigrina* (Rayburn et al. 2004; Ramakrishnan and DeSaer 2011), sex modulation (Lürling et al. 2005; Quinn et al. 2008), molting deformities (Ferrari et al. 2003, 2004; Fatta-Kassinos et al. 2011), and slower maturation (Brodie 2010; Dietrich et al. 2010) in water flea *Daphnia* spp. These effects may be caused by CBZ inhibiting foraging capacity and food assimilation (Dietrich et al. 2010), which changes the energy budget of the affected organism and ultimately affects metabolism, reproduction, and growth (Muller et al. 2010; Gust et al. 2011; Meredith-Williams et al. 2012).

Ceriodaphnia dubia is a common invertebrate biological model that plays an important role in freshwater ecosystems as a filter feeder and also serves as a food source for higher organisms (Norberg and Mount 1985; Knight and Waller 1992). Cladocerans are sensitive to a wide range of toxicants (Cleuvers 2004), and multigenerational studies using these organisms can elucidate long-term effects on sensitive life history traits at sublethal concentrations (Brennan et al. 2006; Clubbs and Brooks 2007; Ramirez et al. 2007; Haeba et al. 2008; Brooks et al. 2009; Massarin et al. 2010; Phyu et al. 2011), which may improve the understanding of potential effects in aquatic systems.

Although ample numbers of ecotoxicological studies (Klüttgen et al. 1996; Boxall 2004; Oetken et al. 2005; Ankley et al. 2007; Kim et al. 2007; Rodgher and Espíndol 2008) have focused on the long-term effects of toxicants at sublethal concentrations, relatively little research has examined effects of chronic exposure of anticonvulsant drugs to multiple generations of planktonic invertebrates. Our research goal was to understand how, and if, sublethal CBZ exposure affects key life history characteristics over multiple *C. dubia* generations. Because CBZ has recently been shown to alter histone deacetylases and ultimately gene transcription, concern over potential multigenenerational effects may be warranted (Beutler et al. 2005). We assessed parameters, such as reproduction, growth, and respiration, to evaluate the sensitivity of these processes and their importance in the ecological risk assessment of CBZ.

Materials and Methods

Chemicals

CBZ (5H-dibenzo[b,f]azepine-5-carboxamide; CAS no. 298-46-4) and deuterated CBZ were obtained from Sigma–Aldrich (St. Louis, MO). The relevant physical and chemical properties of CBZ are as follows: chemical purity >99 %; water solubility 17.7 mg/L at 25 °C; log Kow 2.45; and vapor pressure 1.84E-007 mm Hg (25 °C). Formic acid and high-performance liquid chromatography grade (HPLC) methanol were obtained from Fisher Scientific (Houston, TX). Milli-Q water was obtained from the laboratory's own Milli-Q water system (Millipore, Billerica, MA).

Test Organisms

Ceriodaphnia dubia were obtained from Aquatic Biosystems (Fort Collins, CO). Mass cultures of *C. dubia* were maintained at 25 °C in the University of North Texas Aquatic Toxicology Laboratory. A 16:8 hour light-to-dark photoperiod was maintained with a fluorescent light intensity of 980–1030 lux. *C. dubia* were cultured in 500 mL glass jars with reconstituted hard water (RHW) beginning with neonates <24 h old. Neonates from mass cultures were isolated and individually kept in 30 mL clear portion plastic cups. Culture and test organisms were fed once daily with 0.5 mL 20×10^6 cells/mL unicellular green algae *Pseudokirchneriella subcapitata* and 60 µL cerophyl (cereal grass media) suspension. These tests followed standard United States Environmental Protection Agency (USEPA) procedures (United States Environmental Protection Agency 2002a, b) with the exception that the

yeast–cerophyl trout chow feeding suspension was replaced with cerophyl only as recommended by Knight and Waller (1992). Cerophyl extract was prepared weekly by blending 5 g cerophyl/L RHW and filtering the blend through a 40 μm mesh.

Toxicity Test

Experimental Design

Acute Toxicity CBZ exposure bioassays were conducted in a 48 h static-renewal test according to the methodology described in USEPA guidelines (2002). Six nominal concentrations of CBZ (0, 0.625, 1.25, 2.5, 5, and 10 mg/L) were used with four replicates consisting of five organisms each. These experiments were initiated with *C. dubia* neonates <24 h old. The number of living and dead organisms was noted at 24 and 48 h to calculate the EC_{50} value.

Chronic Toxicity Sublethal CBZ nominal concentrations were chosen based on a 7-day range-finding reproduction study; the range of 95 % confidence interval of the EC_{50} value was 246.3–296.8 μg/L. Six nominal concentrations of CBZ (0, 17.5, 35, 70, 140, and 280 μg/L) were used with 10 replicates consisting of 10 organisms in each of 10 cups. All tests were conducted for 14 days, and the end points included reproduction, growth, and productivity (dry weight) for each generation based on the methodology described in USEPA guidelines for static-renewal testing (United States Environmental Protection Agency 2002a, b). The experiments were initiated with <24-hour-old *C. dubia* neonates for all three generations (F0, F1, and F2). End points quantified were reproduction, growth, and respiration. Reproduction was measured as the total number of neonates produced in each brood. For growth, <24-hour-old neonates born on days 0, 7, and 14 of each generation were taken for body size measurement, and adult body length and weight were measured on day 14. Neonate dry weight was measured from every brood produced. Respiration was measured on day 0 neonates. Third-brood neonates were used to start the experiment as well as to start the study of successive F1 and F2 generations. Ten replicate 30 mL cups, each containing 15 mL test solution, were used for each treatment and were renewed daily. Reconstituted hard water was used as diluents and control. Only adult *C. dubia* were transferred to freshly prepared solutions every 24 h. The numbers of neonates produced were recorded daily. United States Environmental Protection Agency (2002a, b) acceptability criteria were met (≥80 % survival of all control organisms and 60 % of surviving control *C. dubia* had produced at least a third brood and an average of 15 neonates/surviving female *C. dubia* in 7 days).

Moderately reconstituted hard water was prepared according to United States Environmental Protection Agency (2002a, b) protocol. Prepared RHW had pH, alkalinity, and hardness levels ranging from 7.4 to 7.8, 57 to 64 mg $CaCO_3$/L, and 80 to 100 mg $CaCO_3$/L, respectively. Reagents were purchased from Fisher Scientific (Pittsburg, PA). Water temperature, dissolved oxygen, conductivity, pH, alkalinity, and hardness were measured and recorded every week for each treatment.

End Point Measurements

To accurately measure neonate weight, several preparatory procedures were undertaken. Neonates were transferred to a glass Petri dish containing 20 mL Milli-Q water for 1 h. Organisms were washed five times with Milli-Q water to clear salt from their gut. Organisms were then kept in preweighed polypropylene 2 mL centrifuge tubes with lid in a freezer at −20 °C. After 4 h, samples were inserted into a lyophilizer to separate organisms from water, salt, and chemicals at −40 °C and high vacuum (200 mbar) for 24 h. Dried samples were kept in a desiccator for half an hour before weighing to the nearest 0.01 mg on an analytical microbalance (H51AR, Mettler Toledo, Columbus, OH). Body length was measured from the apex of the head to the base of the tail spine at the carapace using a digital analyzing system (Axio Vision Rel. 4.7 imaging system camera; Carl Zeiss, Germany) calibrated with a micrometer at 5 × (2.02 μm), 10 × (1.01 μm), and 40 × (0.25 μm) with X- and Y-scaling. Adult sex was identified based on morphological characteristics as described by United States Environmental Protection Agency (1986). Female and male *C. dubia* were characterized under a 60× ocular microscope by the presence of tooth-pecten in the postabdominal claw and extended antennules, respectively.

Ceriodaphia dubia respiration was measured using <24-hour-old third-brood neonates from F0 control female animals in a closed-system respirometer (model 2006; Forma Scientific). Neonates of the same age were placed inside a closed respirometer at 25 ± 1 °C in 178.06 μL RHW. Neonates were acclimatized in a 13.6 or 264.6 μg/L CBZ solution for 1 h before placement in the respirometer. Three chambers, each with 30–40 offspring and one blank chamber, were monitored for 1 h. Eight sets of control neonates, six sets of neonates at 13.6 μg/L and six sets of neonates at 264.6 μg/L, were measured in this manner. Organisms were acclimatized at normoxic oxygen conditions (P_{O2amb} = 4.18–4.25 kPa). The respiratory medium consisted of RHW to serve as the medium of *Ceriodaphnia* culture. The chamber was cleaned with 70 % ethanol after each use. For calibration, the medium in the chamber was equilibrated to 100 % oxygen saturation. The optical sensor was calibrated using sterilized clean medium and by

flushing the chamber with oxygen. The decrease in oxygen partial pressure associated with respiration was recorded for 60 min. The respiratory activity of the organisms and dependence on P_{O2amb} was determined by monitoring the decrease in oxygen concentration. The oxygen consumption rate at different P_{O2amb} was obtained from the decrease in oxygen concentration per increment of time. The mass specific oxygen consumption rate MO_2 (μL/h mg) was obtained by dividing the whole-animal oxygen consumption rate MO_2 (μL/h) by the dry weight (mg) of the organisms.

Chemical Preparation/Quantification

Ten milligrams of CBZ/L stock solution was prepared in reconstituted hard water at 70 °C by heating, stirring, and sonicating for solution for 5 h. Based on a range-finding study, the following concentrations were used as nominal concentrations for the test: 17.5, 35, 70, 140, and 280 μg/L. The test concentrations were prepared by serial dilution of the stock solution. Once each week, two composite chemical samples from each treatment were taken immediately before addition of algae and cerophyl and then again 24 h after redosing. Therefore, altogether eight samples were analyzed for each CBZ concentration before exposure, and seven samples were analyzed after 24 h of exposure. Methods followed those previously published by Overturf et al. (2011). Briefly, CBZ-d2 was used as labeled internal standard for all samples to quantify the compound using LC–tandem mass spectroscopy (LC–MS/MS). Samples were analyzed with a Waters 2695 separations module coupled with a Waters Quattro Ultima mass spectrometer running in electrospray positive ionization mode according to the method developed by Miao and Metcalfe (2003). The CBZ initial method mobile phase was 30:70 Milli-Q:MeOH with 0.1 % formic acid mixture running at a flow rate of 0.2 mL/min. The capillary voltage, cone voltage, source temperature, and desolvation temperature were 4 kV, 60 V, 120 and 350 °C, respectively. The mass spectrometer was set on multiple reaction monitoring mode, and the mass transitions of CBZ and CBZ-d2 were 237 → 194 and 239 → 196, respectively. A nine-point for CBZ and standard calibration curve was developed based on the range-finding concentrations (3.9–1,000 μg/L). Quality-control samples were taken to confirm precision (≤20 %), accuracy (≥80 %), and reliability of the study using method blanks, matrix spikes, and sample spikes.

Statistical Analysis

Data were analyzed using SAS 9.2 (SAS, Carey, NC). The acute toxicity test was analyzed using Fisher's exact test to compare treatment group survival to control survival. Normality and homogeneity of variance were checked for all study end point parameters using Shapiro–Wilk normality and Bartlett's tests, respectively. One-way analysis of variance (ANOVA) with Tukey's Studentized Range (HSD) post hoc tests and minimum significance difference (MSD) were calculated to determine significant differences of measured concentrations relative to controls on survival, reproduction, body length, dry weight, and respiration rate. When parametric assumptions for normality were not met, nonparametric Kruskal–Wallis test was conducted. In all tests, the significance level used was $\alpha = 0.05$ to calculate no observed-effect concentration (NOEC) and lowest observed-effect concentration (LOEC) values. Effective concentration (EC_5, EC_{10}, EC_{20}, and EC_{50}) values were calculated using TRAP (version 1.21A; http://www.epa.gov/med/Prods_Pubs/trap.htm by Russell Erickson). GraphPad Prism 5 (GraphPad Software, CA) software was used for graphical representation.

Results

CBZ was not detected in control dilution water samples. Limit of detection for CBZ with LC-MS/MS was 0.97 μg/L. The median observed CBZ concentrations before exposure for the nominal treatment levels of 17.5, 35, 70, 140 and 280 μg/L were 13.6 ± 5.26, 40.0 ± 5.15, 104.0 ± 7.94, 196.7 ± 9.07 and 264.6 ± 6.58 (median, μg/L ± SD), respectively (Table 1).The percent recovery of matrix and sample spikes was 91.5 ± 4.5 % ($n = 3$) and 107.6 ± 3.6 % ($n = 3$) respectively. CBZ degradation ranged from 51 to 74 % loss. We assume the loss of CBZ is due to absorption in the exposure cups. This includes absorption by *C. dubia*, algae, and losses while processing the samples.

The 48 h CBZ acute toxicity to *C. dubia* resulted in a median lethal concentration of 7.07 mg/L. Analysis of three-brood production among control organisms in three generations showed no statistical differences ($F_{2, 27} = 0.22$, $p = 0.807$) (MSD 3.4 or 12.3 %). Results indicate that *C. dubia* produced three broods with an average of 27 offspring over 7 days in the control and 6–8 broods over the initial 2 weeks of the study. For the 7-day chronic toxicity test, the NOEC and LOEC values for reproduction on the F0 generation were 196.7 and 264.6 μg/L, respectively, and similar patterns were observed over successive F1 and F2 generations (Table 2). Overall, ANOVA results showed that neonate production over three broods decreased significantly at 264.6 μg/L CBZ treatment in the F0 ($F_{5, 54} = 7.6$, $p < 0.0001$), F1 ($F_{5, 54} = 3.9$, $p = 0.004$), and F2 ($F_{5, 54} = 3.8$, $p < 0.005$) generations compared with the control (MSD in F0, F1 and F2 were 5.2 or 14.8 %, 5.5 or 19.6 %, and 2.4 or 8.5 % of the control, respectively).

Table 1 Summary of CBZ concentrations of LC–MS/MS detection in water samples before and after 24 h of exposure

Nominal concentration (μg/L)	Before exposure Median measured concentration (μg/L) ± SD ($n = 8$)	After exposure (24 h) Median measured concentration (μg/L) ± SD ($n = 7$)	Loss (%)
Control	<LOD	<LOD	–
17.5	13.6 ± 5.26	6.7 ± 2.17	50.7
35.0	40.0 ± 5.15	16.0 ± 3.85	59.9
70.0	104.0 ± 7.94	26.7 ± 4.82	74.3
140.0	196.7 ± 9.07	78.0 ± 7.19	60.3
280.0	264.6 ± 6.58	99.7 ± 7.34	62.3

Analysis of total brood production at 2 weeks among control organisms in three generations showed no statistically significant differences ($F_{2,\ 27} = 0.01$, $p = 0.988$) (MSD 11.97 or 13.3 %). Chronic toxicity effects of CBZ were compared for the total number of offspring born over 2 weeks relative to the control showed EC_{50} and EC_{20} values of 365.3 and 162.5 μg/L, respectively (Fig. 1a; Table S1 in Supplementary Material) in the F0 generation. The NOEC and LOEC values were 104.0 and 196.7 μg/L, respectively, and similar patterns were observed in the F1 generation (Table 2). However, in the F2 generation, NOEC and LOEC values for fecundity were 196.7 and 264.6 μg/L, respectively. Overall, ANOVA results showed that neonate production over 2 weeks decreased significantly at the 196.7 μg/L CBZ treatment in the F0 ($F_{5,\ 54} = 17.1$, $p < 0.0001$) and F1 ($F_{5,\ 54} = 10.8$, $p < 0.0001$) generations, but no significant difference was observed relative to control in the rest of the treatments for the first two generations. The EC_{50} value was 360.4 μg/L, which is slightly lower than that in the F0 generation (Fig. 1b; Table S1 in Supplementary Material). In the F2 generation, the EC_{50} and EC_{20} values were 316.2 and 167.9 μg/L, respectively (Fig. 1c; Table S1 in Supplementary Material). In addition, effect concentrations were more substantial on the F2 generation than on the F0 and F1 generations, as shown in Fig. 2, due to steep slope at higher concentration (MSDs in F0, F1, and F2 were 15.76 or 19.5 %, 17.6 or 21.7 %, and 22.3 or 24.8 % of the control, respectively).

First and second generations were not statistically different with respect to time to first reproduction. Median time to first brood production was significantly delayed ($F_{5,\ 51} = 5.34$, $p = 0.003$) at the 264.6 μg/L CBZ treatment compared with the control in the F2 generation, indicating slower maturation (Table 2). The time to first reproduction ranged from 5 to 8 days at 264.6 μg/L.

A significant decrease on adult body length was observed in the F2 generation at 264.6 μg/L CBZ treatment ($F_{5,\ 50} = 2.99$, $p < 0.0195$). The EC_{50} and EC_{20} values were 559.8 and 348.3 μg/L, respectively (Fig. 3a; Table S1 in Supplementary Material). Overall, no significant differences were observed in the F0 generation relative to the control, and a similar pattern was also observed in the F1 generation (Fig. 3a–c). Offspring body lengths measured in

Table 2 Summary of LOEC and NOEC values for *C. dubia* exposed to CBZ over three generations

End points/generation	F0			F1			F2		
	LOEC (μg/L)	NOEC (μg/L)	*F* ratio, *p* value	LOEC (μg/L)	NOEC (μg/L)	*F* ratoi, *p* value	LOEC (μg/L)	NOEC (μg/L)	*F* ratio, *p* value
Fecundity[a]									
7 d, 3 broods	264.6	196.7	$F_{5,\ 54} = 7.6$, **$p < 0.0001$**	264.6	196.7	$F_{5,\ 54} = 3.9$, **$p = 0.004$**	264.6	196.7	$F_{5,\ 54} = 3.8$, **$p = 0.005$**
14 d, 1–7 broods	196.7	104.0	$F_{5,\ 54} = 17.1$, **$p < 0.0001$**	196.7	104.0	$F_{5,\ 54} = 10.8$, **$p < 0.0001$**	264.6	196.7	$F_{5,\ 54} = 10.7$, **$p < 0.0001$**
Age at first reproduction (d)[b]	≥264.6	264.6	$F_{5,\ 54} = 0.8$, $p = 0.977$	≥264.6	264.6	$F_{5,\ 54} = 0.2$, $p = 0.924$	264.6	196.7	$F_{5,\ 51} = 5.3$, **$p = 0.003$**
[a]Growth									
Neonate body length (mm)	≥264.6	264.6	$F_{5,\ 354} = 1.1$, $p = 0.328$	≥264.6	264.6	$F_{5,\ 354} = 0.73$, $p = 0.599$	≥264.6	264.6	$F_{5,\ 340} = 0.42$, $p = 0.834$
Adult body length (mm)	≥264.6	264.6	$F_{5,\ 51} = 0.09$, $p = 0.993$	≥264.6	264.6	$F_{5,\ 51} = 0.73$, $p = 0.603$	264.6	196.7	$F_{5,\ 50} = 2.99$, **$p = 0.019$**
Neonate dry weight (μg)	≥264.6	264.6	$F_{5,\ 60} = 1.4$, $p = 0.241$	196.7	104.0	$F_{5,\ 60} = 3.3$, **$p = 0.010$**	264.6	196.7	$F_{5,\ 59} = 4.3$, **$p = 0.002$**

[a] Parametric ANOVA, Tukey's multiple comparison test, and minimum significance difference at $\alpha = 0.05$

[b] Kruskal–Wallis nonparametric ANOVA, Dunn's multiple comparison, $\alpha = 0.05$

Statistically significant values ($p < 0.05$) are in bold

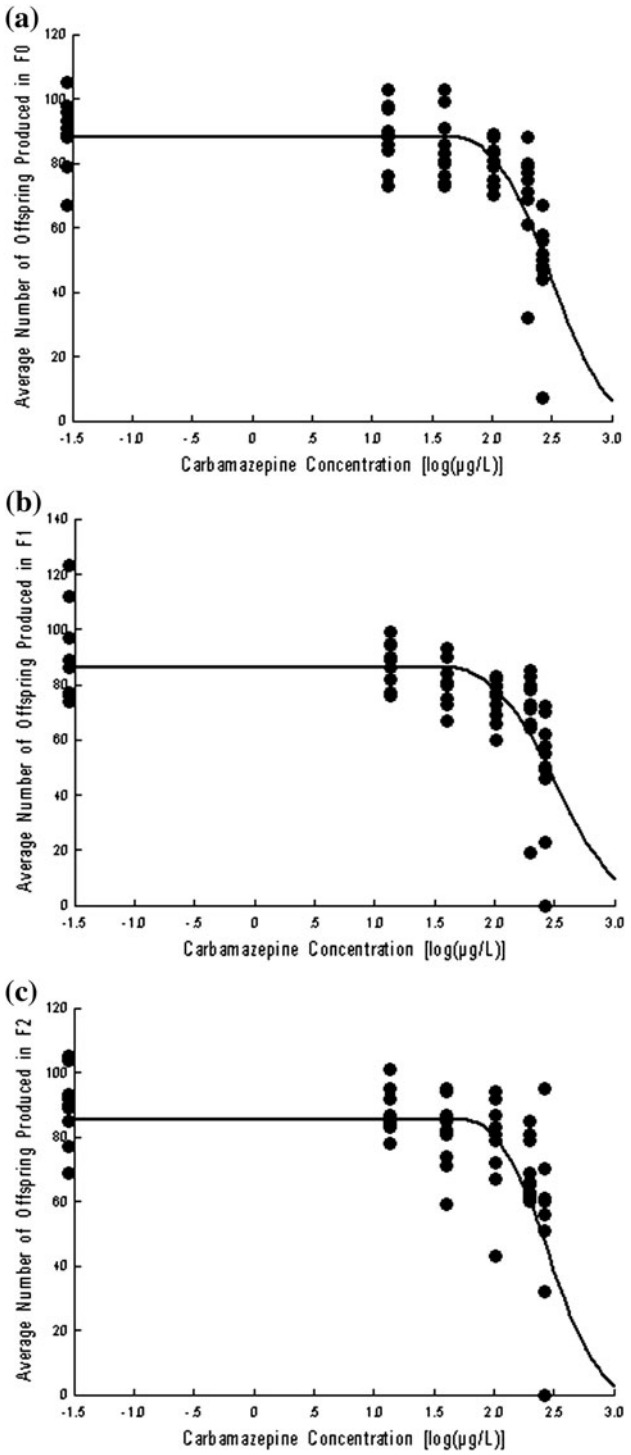

Fig. 1 Average number of offsprings produced in two weeks by *C. dubia* exposed to CBZ in **a** the F0 generation, **b** the F1 generation, and **c** the F2 generation

the first, third, and seventh brood, born on days 4, 7, and 14, respectively, showed no significant difference relative to the control (see Table 2), indicating that CBZ did not influence body length in *C. dubia* offspring. However, there was a slight stimulatory effect on offspring body

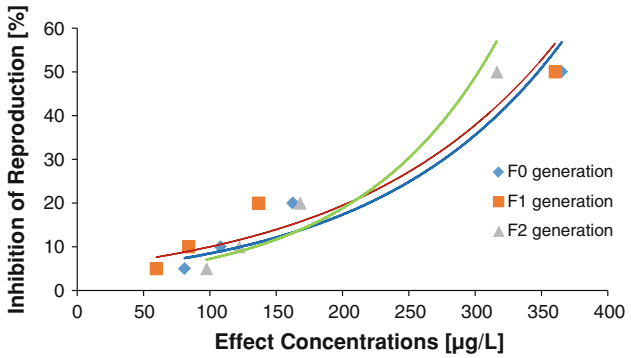

Fig. 2 Measured effect concentrations (EC_x) of CBZ exposure on reproductive output of *C. dubia* over three generations

length measured at 196.7 and 264.6 μg/L in the F3 generation, although the differences were not statistically significant.

No significant differences were observed in the dry weights of neonates exposed to CBZ relative to the control in the F0 generation. However, mean neonate biomass production was slightly greater at 40.0 μg/L than for the control, but a significant decrease of neonate dry weight was observed in successive generations. The EC_{50} and EC_{20} values were 377.8 and 197.6 μg/L, respectively (Fig. 4a; Table S1 in Supplementary Material). In the F1 generation, a significant decrease of biomass production was observed at CBZ concentrations ≥196.7 μg/L compared with the control ($F_{5,60} = 3.33$, $p < 0.0101$). Similarly, in the F2 generation, neonate dry weight was decreased significantly at 264.6 μg/L ($F_{5,59} = 3.74$, $p < 0.0052$). Overall, effect concentrations were not substantially different among three generations as shown in Fig. 4a–c. However, a decrease in adult dry weight was observed as CBZ concentration increased. Adult dry weight increased at 13.6 μg/L in all three generations studied, but it decreased relative to the control at greater concentrations (Table S2 in Supplementary Material). No male production was found in any control, and 3 of 10 adults were not able to reproduce during 2 weeks on their third generation at 264.6 μg/L (Table S3 in Supplementary Material).

The whole-animal oxygen consumption rate (mO_2) increased with CBZ concentration. Significant differences were observed between the control and increased concentration of CBZ at 264.6 μg/L ($F_{2,17} = 5.19$, $p = 0.017$) (Fig. 5). The increased metabolic rate may be due to *C. dubia* neonates using significant amounts of energy to attain tolerance to or detoxify CBZ.

Discussion

The effects of pharmaceuticals on aquatic ecosystems are being widely discussed. The current study was intended to establish a baseline for chronic effects of CBZ on multiple

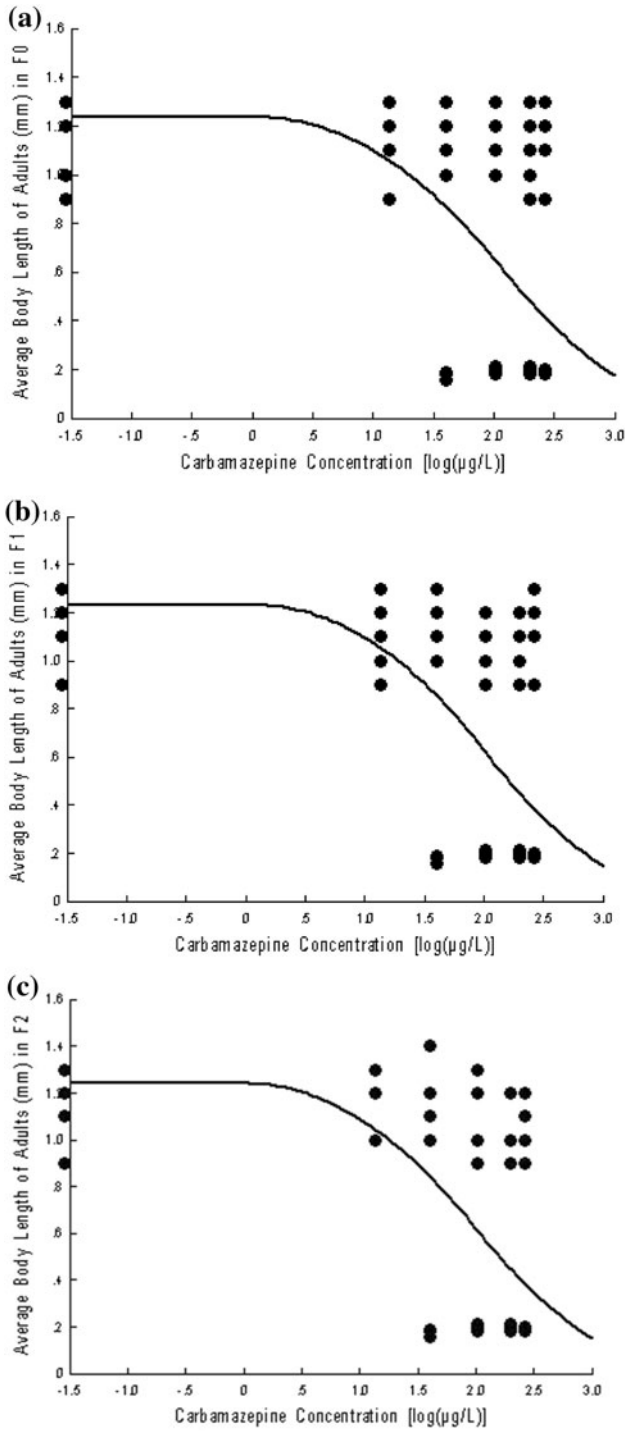

Fig. 3 Average adult body length of *C. dubia* exposed to CBZ on **a** the F0 generation, **b** the F1 generation, and **c** the F2 generation

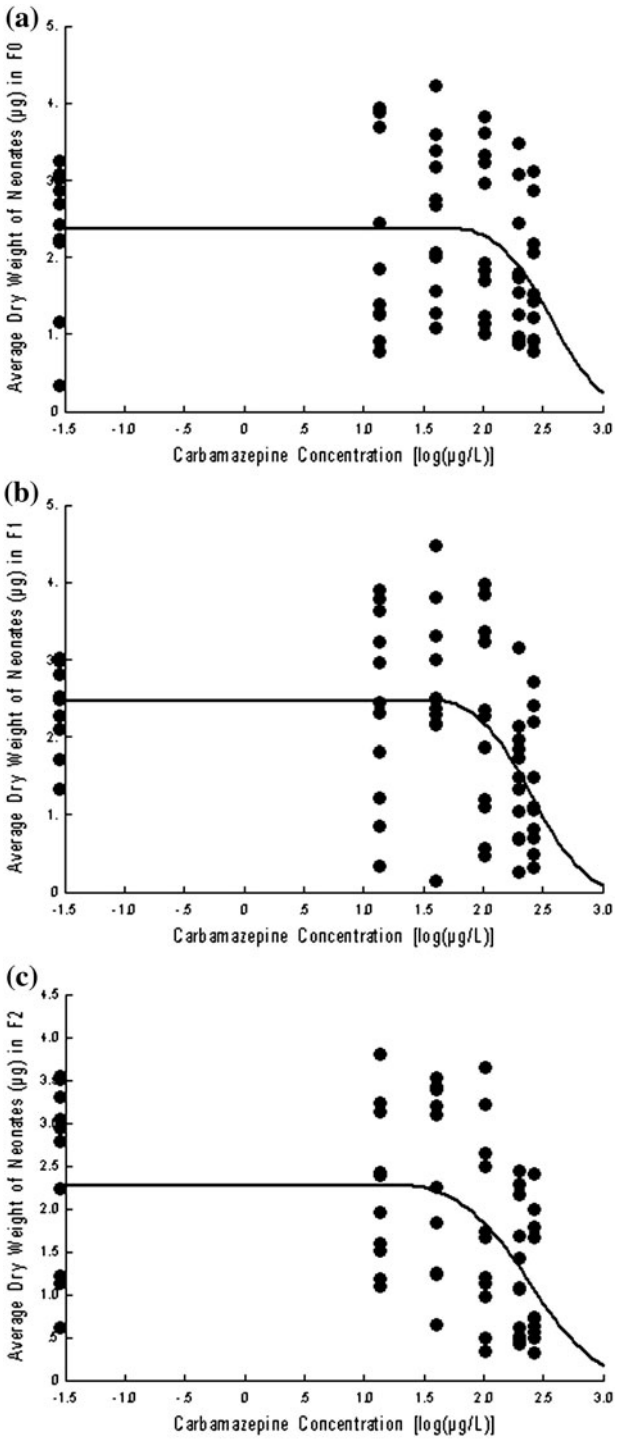

Fig. 4 Average dry weight of neonates of *C. dubia* exposed to CBZ on **a** the F0 generation, **b** the F1 generation, and **c** the F2 generation

generations of *C. dubia*. The mode of action of CBZ on aquatic invertebrates is largely unknown, but it may relate to Na^+ channels blockade as in mammals. However, quantification of individual physiological responses under environmental stress may help scientists better understand the ecotoxicological risks of CBZ. Although changes in fecundity, growth, and metabolic rate can lead to long-term population effects, we only observed meaningful changes to these parameters at CBZ concentrations that were 100× greater than those found in the environment. In addition, our study showed that results over three successive

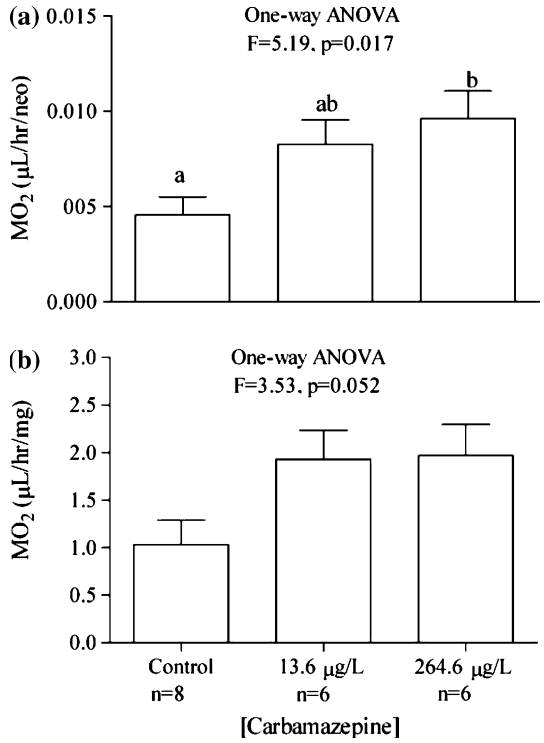

Fig. 5 Respiration rate of *C. dubia* neonates (mean ± SE) exposed to CBZ. **A** Respiration rate per neonate. **B** Mass-specific respiration rate. Lower-case letters "*a*" and "*b*" indicate statistically significant differences from the control treatment (parametric ANOVA, Tukey's multiple comparison test, and minimum significance difference at $\alpha = 0.05$)

generations are not different than what one would gain testing simply the F0 generation.

Brood production over 2 weeks was significantly decreased at 196.7 µg/L in the F0 and F1 generations, but in the F3 generation, neonate production decreased significantly only at the highest concentration (264.6 µg/L), indicating that these organisms may have acquired tolerance by way of physiological acclimatization or developed resistance when exposed to sublethal concentrations. CBZ has recently been shown to significantly decrease glutamate oxaloacetate transaminase activity in fish liver and muscle from 7 days through 35 days of CBZ exposure (Malarvizhi et al. 2012). Dietrich et al. (2010) elucidated physiological acclimatization of *D. magna* in CBZ exposure from the second to sixth generation. Lürling et al. (2005) observed a significant increase in neonate production over three broods in *D. pulex* at 1 µg/L CBZ and a decrease in reproduction at 200 µg/L CBZ, although the difference was not significantly different from the control. Furthermore, Boxall (2004) argued that long-term chemical exposure could lead to physiological resistance in nontarget organisms.

There is evidence that CBZ affects invertebrates at sublethal concentrations of 0.236–200 µg/L (Cleuvers 2004; Lürling et al. 2005; van den Brandhof and Montforts 2010; Dietrich et al. 2010; Contardo-Jara et al. 2011). Contardo-Jara et al. (2011) found that CBZ bioaccumulates in nontarget organisms (*D. polymorpha*) and causes oxidative stress and protein damage at the lowest concentration of 0.236 µg/L. Their study showed that CBZ exposure significantly downregulated mRNA levels of hsp70, and the bioconcentration factor was significantly increased by 17 (day 1) to 90 (day 7) on mussel tissues. Those investigators surmised that the cell damage they measured could lead to effects on reproduction and growth.

In a study by Lürling et al. (2005), the age at first reproduction in *D. pulex* was found to be significantly delayed at 200 µg/L CBZ exposure. The current study shows that the median day to release of the first brood was significantly delayed at 264.6 µg/L in the F3, indicating a delay in maturation. Furthermore, Dietrich et al. (2010) reported a significant delay in the first brood production of *D. magna* in the F0 and F2 generations exposed to 0.5 µg/L CBZ, suggesting potential chronic effects due to the chemical. This inference was also supported by evidence from Calabrese and Baldwin (2003). In contrast, Oetken et al. (2005) found increased emergence of *C. riparius* at low concentrations of CBZ but increased emergence at high concentrations, indicating that the hormetic process is an adaptive response that protects organisms from toxic stress.

Our results indicated that CBZ did not exert any effects on neonate body length, which is consistent with the findings of Lürling et al. (2005) who observed that the length of treated *D. pulex* neonates was not significantly different compared to the control. CBZ did not exert any effects on neonate body length. However, a slight stimulatory effect on offspring body length was observed at 196.7 and 264.6 µg/L in the F2 generations, although the differences were not significant. In contrast, Dietrich et al. (2010) observed significantly increased neonate body length in the first and the fifth generations but no effects in the rest of the generations studied. Dietrich et al. (2010) also reported observable effects on adult *D. magna* body length as they acclimated to exposures of CBZ alone and mixtures of CBZ and other pharmaceuticals. Dry weight was significantly decreased at the LOEC value of 196.7 µg/L in the F1 generation. Overturf et al. (2011) observed slight increases in dry weight of *Pimephales promelas* larvae at a sublethal CBZ concentration; however, the differences were not significant. The current study showed that reproductive parameters are sensitive to CBZ exposure because the proportion of the male offspring (3 of 10 male animals) started to increase during the F2 generation at the 264.6 µg/L treatment. Olmstead and LeBlanc (2001) reported that cladoceran parthenogenetic asexual reproduction switched to sexual reproduction due to either genetic exchange or diapausing juvenile hormone due to

environmental stressors. The production of male animals has been reported in several studies resulting from environmental factors and stress due to toxicants (United States Environmental Protection Agency 1991; Shurin and Dodson 1997; Dodson et al. 1999; Olmstead and LeBlanc 2000; Haeba et al. 2008). Baer et al. (2009) showed that sewage effluents modulated male offspring production of *D. magna*, which is in agreement with findings that these chemicals stimulated male production due to strong juvenoid agonist activity (Wang et al. 2005). Haeba et al. (2008) showed a significant increase in *D. magna* male offspring exposed to diclofol at 0.1 mg/L. They also showed suppression of neonate production, decreased in maternal size, and delayed maturation of *D. magna* exposed to fluatamide at 1 mg/L. Furthermore, Olmstead and LeBlanc (2000) showed development of secondary sex characteristics, i.e., male formation on *D. magna*, exposed to endocrine-disrupting chemicals. Nentwig et al. (2004) showed impaired development from the larval to the pupal stage in *C. riparius* at 10 mg CBZ/kg dw.

Respiration, a surrogate of metabolism, significantly increased at 264.6 µg CBZ/L compared with the control. Metabolic increase may be due to *C. dubia* neonates using significant amounts of energy to attain tolerance or detoxify CBZ, thus leaving less energy for reproduction. Maintenance costs are generally estimated by measurements of organism respiration rate, which can be fitted with reproductive success and body size in dynamic energy budget models (Kooijman 2000; Muller et al. 2010; Nisbet et al. 2010). Limited studies are available on the respiratory effects of antidepressant exposure to aquatic organisms. However, CBZ acts as an inhibitor of histone deacetylases, induction of gene transcription, and Na^+ channel blockage (Beutler et al. 2005), which might be associated with high energetic costs in the metabolic process in cladocerans. Quinn et al. (2004) showed a significant increase in the oxidative metabolic activity in *Hydra attenuate* exposed to CBZ. Their study showed induction of heme oxidase, lipid peroxidase, and glutathione *S*-transferase at 6 µM (1.4176 mg/L) CBZ exposure. Many studies have also showed that resistance to toxic chemicals in aquatic organisms depends on the mode of action of the toxic chemicals and the susceptibilities of nontarget organisms (Rose et al. 2002; Heckmann et al. 2007). Dietrich et al. (2010), however, argued that some aquatic organisms cannot develop resistance toward the long-term exposure to toxic chemicals over several generations due to the necessity of high energy acquisition and expenditure (Muller et al. 2010).

There is potential for additive toxic effects between CBZ and other chemicals found in the environment. Cleuvers (2003), for example, reported a strong additive effect between CBZ (>100 mg/L) and clofibrinic acid (72 mg/L) on *D. magna* immobilization: 95 % of *Daphnia* were immobilized in a mixture of these chemicals compared with 16 and 1 % immobilized with exposure to CBZ and clofibrinic acid separately. Differences in fecundity, growth, and respiration between controls and the treatment groups (13.6, 40.0, and 104.0 µg/L) were not always significant; however, differences indicated potential chronic effects of CBZ over generations.

Our study determined that testing over three successive generations produces results that are not different from those that one would gain by testing simply the F0 generation. Exposure levels in the current study were much higher than what would likely be seen in the environment. Our results suggest that high concentrations of CBZ interfere with various life-cycle processes, such as survival, reproduction, growth, and respiration. Reproductive impairment was observed at sublethal concentrations, which suggests long-term sensitivity to the compound. *C. dubia* shows no observable threshold effects at low chemical concentrations. In our study, the individual effects of CBZ exposure on all observed life-cycle parameters occurred at levels approximately 100 times that of environmentally relevant concentrations, i.e. 196.7 µg/L CBZ compared to 2.1 µg/L in surface water as observed by Ferrari et al. (2003). Recently, Zhang et al. (2012) reported that CBZ significantly inhibited growth and chlorophyll content of algae (*Scenedesmus obliquus* and *Chlorella pyrenoidosa*) at a 1 mg/L concentration. However, Andreozzi et al. (2002) reported that CBZ neither had a significant effect on *P. subcapitata* growth at environmentally relevant concentrations nor that it bioaccumulated.

In the current study, neonate production was significantly decreased for the F0 generation (Fig. 2). The trend remained consistent through the F2 generation, although in the F2 generation, the trend was not statistically significant at <196.7 µg/L. Our results indicate that there is a trade-off between growth, reproduction, and respiration to maintain reproductive output. The reason for this could be that organism's exhibit tolerance to the contaminant or become physiologically acclimated. The lowered reproductive output is evidence for higher energy needs for maintenance. Such transgenerational effects at sublethal concentrations may involve development of a genetically resistant population over the long term. Metabolic rate is sufficiently higher with higher chemical concentrations, leading to significantly decreased fecundity and body size over three generations. Identifying the mode of action of CBZ will be essential to developing models that enable one to predict the toxicity of compounds and extrapolate their consequences to the population level. Research needs include linking metabolic cost of exposure to population life-cycle traits. Aquatic organisms live in a soup of chemicals to which they are exposed throughout their lifetimes and over

generations. Confounding factors, such as mixture of chemicals and chemical uptake by way of food items, has been shown to alter chemical bioavailability (e.g., Cleuvers 2004). There is much yet to be learned pertaining to the effects of metabolites and chemical mixtures to nontarget organisms. Such scenarios are even more critical in cases where CBZ may be acting together with other co-occurring compounds, potentially causing long-term effects. Future studies are also needed that apply the modeling approach on effects of CBZ metabolites and their co-occurring chemical mixtures at the molecular level with more environmentally realistic concentrations over multiple generations.

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Conflict of interest The use of trade, firm, or brand names in this article is for identification purposes only and does not constitute endorsement by the authors.

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