Arch Environ Contam Toxicol (2009) 56:450–458
DOI 10.1007/s00244-008-9231-5

Effects of Sublethal Chronic Copper Exposure on the Growth and Reproductive Success of the Florida Apple Snail (*Pomacea paludosa*)

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Received: 2 June 2008 / Accepted: 1 September 2008 / Published online: 10 October 2008

Abstract Florida apple snails (*Pomacea paludosa*) were exposed to three concentrations of copper (Cu), in water (8 μg/L, 16 μg/L, 24 μg/L), for one generation to examine uptake and the effects on survival, growth, and reproduction of the F_0 generation and survival, growth, and whole body Cu of the F_1 generation. During a 9-month Cu exposure, apple snails exposed to 8–16 μg/L Cu had high Cu accumulation (whole body, foot, viscera, and shell) and significantly reduced clutch production (8–16 μg/L) and egg hatching (16 μg/L). Apple snails exposed to the 24 μg/L Cu had low survival and the treatment was therefore terminated. Concentrations of minerals (Na^+, K^+, Mg^{2+}, Ca^{2+}) in tissues were maintained regardless of Cu exposure, but the distribution of Cu in the body of snails differed, depending on exposure concentrations. Higher exposure concentrations resulted in a greater percentage of Cu accumulated in the viscera of the snail. Copper exposure to the F_0 generation did not affect the survival, growth, or whole body Cu concentrations in the F_1 generation. These finding are significant, given the importance of the Florida apple snail in the Everglades food chain. Changes in the abundance of apple snail populations, as a result of Cu exposure, could ultimately affect foraging success of predators.

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A century of continuous copper (Cu) use in south Florida, as an herbicide, fungicide, and soil amendment, has resulted in elevated Cu concentrations in soils, especially in and around citrus agriculture areas, where it has been used extensively (Alva et al. 1995). In accordance with the Comprehensive Everglades Restoration Plan (CERP), large areas of agricultural lands have been acquired by the state of Florida and the federal government to create buffer zones, water-storage reservoirs, and wetlands (US Department of the Interior 2001–2005); however, conversion of Cu-contaminated soils from dry aerobic environments to inundated anaerobic sediments will promote desorption of residual Cu from the soils to the overlying aquatic environment (Hoang et al. 2008a). Hoang et al. (2008a) reported that soils collected from south Florida citrus groves contained Cu concentrations up to 234 mg/kg Cu dry weight (dw), which resulted in overlying water Cu concentrations (dissolved) of 308 ug/L, after being flooded.

Exposure to high concentrations of Cu has adverse effects on freshwater organisms, including the Florida apple snail (*Pomacea paludosa*), a prosobranch gastropod that is the main food source of the federally endangered snail kite (*Rostrhamus sociabilis plumbeus*) and part of the diet of many other organisms (Sharfstein and Steinman 2001). Recent studies have shown that Cu is acutely toxic to *P. paludosa*, especially in early life stages (96-h LC_{50} range = 34–44 μg/L Cu) and at low pH and dissolved organic carbon (DOC) concentrations (96-h LC_{50} = 19 μg/L Cu at pH 5.5, 0.2 mg/L DOC) (Rogevich et al. 2008), and it will significantly affect growth in juvenile snails (Hoang et al. 2008a). Research also indicates that *P. paludosa* can accumulate Cu from soil, water, and dietary routes of exposure, which might have adverse effects on its predators (Hoang et al. 2008b).

Copper toxicity to freshwater organisms has been studied extensively (Erickson et al. 1996; Howarth and Sprague 1978; Miller and Mackay 1980; Pagenkopf 1983); however, the effects of long-term, chronic Cu exposure are less frequently reported. McKim and Benoit (1971) reported that brook trout (*Salvelinus fontinalis*) had decreased egg production and hatching when exposed to 32.5 μg/L Cu but not at lower concentrations. Conversely, chronic exposure of Cu (1.9–31.8 μg/L) to five cladoceran species showed that whereas Cu treatments reduced growth in all of the species, it did not affect clutch size in any of the species studied (Koivisto et al. 1992). These conflicting data make it difficult to predict whether long-term Cu exposure will adversely affect reproduction in *P. paludosa*.

Although no laboratory studies are available examining the long-term, chronic effects of Cu on *P. paludosa*, it is apparent that snails inhabit areas with high Cu concentrations and accumulate Cu. Frakes et al. (2008) reported that adult apple snails collected from a flooded active citrus grove in Florida contained whole body Cu concentrations as high as 336 mg/kg. This is 10-fold higher than the lethal Cu body burden (30 mg/kg) in adult apple snails in a 96-h acute Cu toxicity study (Rogevich et al. 2008) and similar to concentrations that adversely affected growth and survival in apple snails in a 28-day soil/water system (Hoang et al. 2008a). This indicates that long-term, chronic exposure in the environment results in high concentrations of Cu in snail tissues and that snails might employ detoxification mechanisms (e.g., sequestration via phosphate granules) to compensate and ameliorate chronic toxicity (Mason and Nott 1981). However, it is unclear whether these snails reproduce and produce viable offspring, and if so, whether Cu will transfer from the parental generation (F_0) to their offspring (F_1) as a means of elimination, as observed for Cd, Se, and Hg in other freshwater invertebrates (*Daphnia magna)* (Lam and Wang 2006). Cu exposure has been shown to reduce internal concentrations of other minerals (i.e., Ca^{2+}, Mg^{2+}) via competitive interactions at the site of uptake (Lauren and McDonald 1985; Santore et al. 2001). Mineral concentrations, which are required for maintenance of internal homeostasis and are important in several aspects of reproduction (i.e., shell calcification) (Turner and McCabe 1990), could also be altered by long-term exposure to Cu.

Therefore, the purpose of this study was to determine the effects of long-term, chronic Cu exposure on the survival, growth, and reproductive success of *P. paludosa* (F_0) exposed to three Cu concentrations and the subsequent hatching, survival, and growth of offspring (F_1). Cu and mineral concentrations in tissues of F_0 and F_1 snails were also examined in this study.

Materials and Methods

The chronic, life-cycle toxicity test methods were adapted from the US Environmental Protection Agency Ecological Effects Test Guideline OPPTS 850.1500 Fish Life Cycle Toxicity (US Environmental Protection Agency, 1996a). *P. paludosa* used in this study were hatched from egg clutches from an in-laboratory culture initiated from egg clutches initially collected from Water Conservation Areas 3A and 2B in Broward and Dade counties (FL) using the techniques described by Corrao et al. (2006). Eggs were hatched under laboratory-controlled conditions.

The study consisted of a 230-day Cu chronic exposure phase, in which snails (F_0) (≤4-day-old) were monitored for growth, Cu and mineral concentrations in tissues, and time until onset of reproduction, followed by a 30-day comparative reproduction phase, in which snails were thinned into mating pairs and examined for clutch production, eggs produced per clutch, hatching and early growth, and whole body Cu in F_1 snails.

Chronic Exposure

Snails were exposed to three Cu treatments (T1 = 8 μg/L, T2 = 16 μg/L, and T3 = 24 μg/L Cu) and a control, each with four replicates. Measured copper concentrations are reported in Table 1. The control was carbon-filtered, ultraviolet-sterilized laboratory freshwater (laboratory freshwater) from the city of North Miami, FL, with pH 7.5 ± 1.0 and with hardness adjusted to 150 mg/L as $CaCO_3$ by adding $CaSO_4 \cdot 2H_2O$ and $MgSO_4$ (Fisher Scientific, Fair Lawn, NJ). Hardness was adjusted to reflect average hardness levels in the Everglades ecosystem (South Florida Water Management District DBHYDRO database 2000–2006; http://www.sfwmd.gov). Cu treatments were prepared by adding Cu stock solution (Cu sulfate pentahydrate; Fisher Scientific, Fair Lawn, NJ) to laboratory freshwater with hardness adjusted to 150 mg/L as $CaCO_3$, as described earlier. At study initiation, ≤4-day-old *P. paludosa* were randomly distributed into 100-L plastic test chambers containing 40 L of test solution until each replicate contained 15 snails. Test chambers were aerated to ensure proper dissolved oxygen (DO) concentrations and were held at 28°C ± 1°C. Snails were fed equal amounts of Cu-free romaine lettuce three times a week.

Treatments and controls were renewed three times per week by replacing 25 L (62.5%) of test solution. Renewal water was made in 120-L plastic tubs, equilibrated for 8 h and distributed evenly to respective replicates. DO, temperature, and pH measurements were made daily. Water hardness, alkalinity, and ammonia were measured weekly. Mortality and behavior observations were made daily.

Table 1 Aqueous Cu exposure and Cu accumulation in adult apple snails after 230 days of exposure

Treatment	Nominal Cu exposure water (μg/L)	Measured Cu exposure water[a] (μg/L)	Foot[a,b] (mg/kg Cu dw)	Viscera[a,b] (mg/kg Cu dw)	Shell[a,b] (mg/kg Cu dw)	Whole body[a,b] (mg/kg Cu dw)
Control	0	3.93 (0.90)	44.14 (9.61)AB	55.46 (24.03)A	0.15 (0.43)AB	21.37 (4.89)A
T1	8	8.84 (1.08)	75.13 (17.18)B	153.88 (62.99)B	3.32 (2.65)B	51.37 (12.62)B
T2	16	16.71 (1.35)	140.51 (39.55)C	396.60 (84.17)C	8.44 (5.92)C	96.03 (11.15)C
T3	24	22.0 (0.76)	NA[c]	NA[c]	NA[c]	NA[c]

Note: Treatments with the same letter (A, B, or C) are not significantly different

[a] Data are mean (standard deviation)

[b] $n = 8$ snails per treatment

[c] Treatment was terminated due to high mortality

Mortality was determined when snails failed to maintain a closed operculum. Water samples were collected at each renewal to verify dissolved Cu and mineral concentrations.

Within 7 days of test initiation, the highest Cu treatment (24 μg/L) had >50% mortality and the treatment was terminated. This is consistent with a previous study in which the 96-h LC_{50} for ≤96-h-old *P. paludosa* was 30.7 μg/L (Rogevich et al. 2008). The remaining treatments and controls had no significant mortalities and treatments were continued until mating occurred and eggs were produced in all control and treatment replicates. On day 60, three snails from each replicate were removed from each treatment and growth measurements (total length, aperture length, diameter, wet weight) were made in accordance with procedures described by Boulding and Hay (1993). When mating was observed, mating pairs were marked as male or female using an indelible marker on the shell, for subsequent identification. Burma grass stems were added to treatment chambers for oviposition and were checked daily. Egg production was recorded daily and stems were removed when eggs were present. Time until first egg clutch production was recorded for each replicate. F_1 snails hatched from the first viable egg clutch produced in each replicate were collected and analyzed for Cu concentrations. The duration of the exposure phase was 230 days. On day 230, all snails were removed from each treatment and sex determination and growth measurements (total length, aperture length, diameter, wet weight) were made for each snail, as described earlier. The largest three females and the largest three males were returned to their respective replicates and were observed for mating and reproduction as described in the Comparative Reproduction section. Sex was determined using the previously mentioned markings, as well as by identifying morphological characteristics. Some of the remaining snails were collected for tissue Cu and cation concentrations (as described below).

Water samples were filtered through a 0.45-mm Gelman nylon mesh, collected in 15-mL graduate polypropylene tubes, and dissolved Cu samples were acidified with concentrated nitric acid to pH 2. Cu and other cations were analyzed using inductively coupled plasma–atomic emission spectrometry (ICP-AES).

Comparative Reproduction

During the comparative reproduction phase, three females and three males in each replicate were observed daily for 30 days. The number of clutches produced and the number of eggs per clutch on Burma grass stems were recorded daily. Clutches were removed from treatment chambers and hatched under laboratory-controlled conditions (26°C ± 1°C and 16:8 light-to-dark photoperiod). Egg clutches were suspended over 1-L beakers filled with laboratory freshwater to hold the F_1 snails as they hatched. Hatching was recorded daily. Ten F_1 snails from the first clutch produced from each replicate (postthinning) were used for growth measurements and 10 snails were collected for whole body Cu analysis.

To measure early growth, 10 F_1 snails (≤24-h-old) from each treatment and control were held in 3 L of laboratory freshwater in glass dishes under laboratory-controlled conditions (26°C ± 1°C and 16:8 light-to-dark photoperiod). Snails were fed equal amounts of romaine lettuce for 28 days. Water was replaced three times a week. Growth was measured on day 28.

Copper Body Burden Analysis

Two F_0 snails from each replicate were collected on day 230 for Cu tissue analysis for a total of 24 snails. Snails were rinsed with EDTA solution and deionized (DI) water to remove Cu bound to the shell surface and were frozen at −20°C until time of analysis. To determine tissue Cu concentrations, each snail was thawed and dissected into three parts (shell, foot, and viscera) following adaptation of a method by Gomot-de Vaufleury and Pihan (2002). Each part of the snail was digested separately with HNO_3 based on US EPA Method 3050B for tissue Cu analysis (US

Environmental Protection Agency 1996b). Cu and cations were analyzed using ICP-AES. Ten F_1 snails were digested and analyzed for Cu and cations using the methods cited earlier.

Data Analysis

Copper concentrations in snails, reproductive end points (days until first clutch, clutch production, number of eggs per clutch), growth, and hatching for treatments were compared using single-factor analysis of variation (ANOVA), followed by Dunnett's procedure (two sided). Data that did not meet the assumptions of normality and homogeneity of variance were log-transformed. All statistical analyses were conducted with SAS version 9.1 (SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Exposure Concentrations and Water Quality

Results of mean exposure Cu concentrations are reported in Table 1. Measured Cu concentrations were consistent with nominal concentrations, fluctuating less than ±10% over the course of the study. The average pH and DO were 7.9 and 8.10 mg/L, respectively. The average ammonia concentration was <0.5 mg/L. The average hardness and alkalinity were 160, 153, and 154 mg/L as $CaCO_3$ and 62, 64, and 64 mg/L as $CaCO_3$, for the control, T1, and T2, respectively. The DOC in laboratory freshwater was 0.3 mg/L.

Copper Concentrations in Adult Snails

In general, whole body Cu concentrations and Cu concentrations in individual parts increased as the Cu exposure concentrations increased. Mean whole body Cu concentrations ranged from 21.37 ± 4.89 mg/kg Cu dw in the control to 96.03 ± 11.15 mg/kg Cu dw in T2 (Table 1). The mean whole body Cu concentration in T1 was significantly greater than in the control ($p < 0.002$) and the mean whole body Cu concentration in T2 was greater than the mean whole body Cu in T1 ($p < 0.000$).

The mean Cu concentrations in the foot ranged from 44.14 ± 9.61 mg/kg Cu dw in the control to 140.51 ± 39.55 mg/kg Cu dw in T2 (Table 1). The mean tissue Cu concentration in T1 was not significantly greater than in the control ($p < 0.066$); however, the mean tissue Cu concentration in T2 was significantly greater than T1 ($p < 0.001$).

The mean Cu concentrations in the viscera ranged from 55.46 ± 24.03 mg/kg Cu dw in the control to 396.60 ± 84.17 mg/kg Cu dw in T2 (Table 1). The mean viscera Cu concentration in T1 was significantly greater than in the control ($p < 0.013$) and the mean viscera Cu concentration in T2 was significantly greater than in T1 ($p < 0.000$).

Shell Cu followed a similar trend as for the foot and viscera. Average Cu concentrations in the shell ranged from 0.15 ± 0.43 mg/kg Cu dw in the control to 8.44 ± 5.92 mg/kg Cu dw in T2 (Table 1). The mean shell Cu concentration in T1 was not significantly greater than in the control ($p < 0.054$); however, the mean shell Cu concentration in T2 was significantly greater than in T1 ($p < 0.022$).

It is evident from these data that the Florida apple snail is able to accumulate significant concentrations of Cu in all portions of their body, including the shell, as a result of long-term exposures to low Cu concentrations (8–16 μg/L) in water. In every treatment, Cu was distributed at the highest concentration in the viscera followed by the tissue, and finally in the shell. These results were similar to distributions found by Hoang et al. (2008b) for adult Florida apple snails for all exposure pathways. Laskowski and Hopkin (1996) found that in the garden snail (*Helix aspersa*), trace metals concentrations (i.e., Zn, Cu, Pb, Cd) in shells of snails exposed for 3 months accounted for <5% of the whole body concentration and most of the accumulated metals were in soft tissues (i.e., foot and viscera).

The current study also showed that under chronic exposure, Cu might accumulate differentially depending on concentrations (Fig. 1). Cu distribution was significantly different in the viscera and foot in snails among the control, T1, and T2 treatments ($p < 0.001$ for tissue Cu and $p < 0.001$ for viscera Cu). Foot Cu concentrations

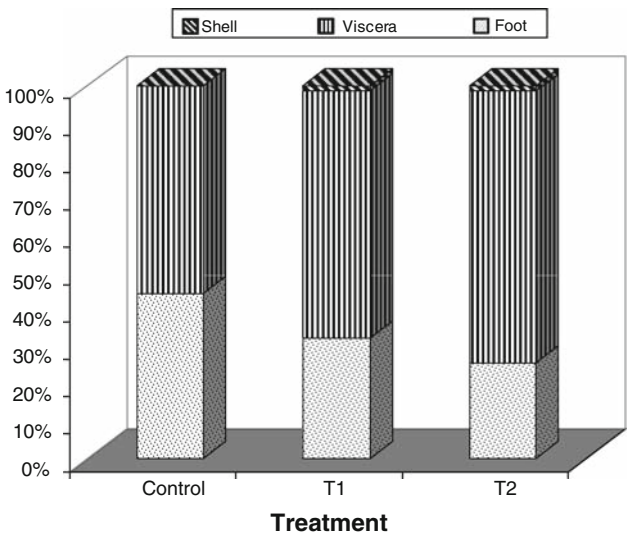

Fig. 1 Distribution of accumulated Cu in adult apple snails after 230 days of aqueous Cu exposure. Data are mean of four replicates; $n = 2$ snails per replicate

accounted for 44.25%, 32.34%, and 25.75% of whole body Cu in the control, T1, and T2, respectively. Viscera Cu concentrations accounted for 55.59%, 66.23%, and 72.70% of whole body Cu in the control, T1, and T2, respectively. Taylor and Anstiss (1999) reported that Cu uptake (whole body and individual tissue) is proportional to exposure concentrations and duration of exposure. Several studies have found that mollusks store excess Cu by sequestering the metals in forms that are biologically unavailable (e.g., as phosphate granules) (Depledge and Rainbow 1990; Mason and Nott 1981; Phillips and Rainbow 1989; Simkiss 1981; Viarengo 1989). Desouky (2006) found that the number of granules in the digestive gland of the pond snail (*Lymnaea stagnalis)* increased after 10 days of exposure to metals (i.e., Al, Zn, Cd) and they accumulated up to 80% of the metals in the viscera (i.e., kidney and digestive gland). Intracellular mineralized granules are located mainly within membrane-limited vacuoles in the digestive gland and kidneys (Soto et al. 1996), which, in the current study, could account for the high accumulation of Cu in the snail viscera relative to other body parts at the higher Cu concentration.

Mineral Concentrations in Adult Snails

Mineral concentrations did not change in any portion of the snail, although Cu concentrations were significantly elevated in the shell, foot, and viscera of snails in both treatments on day 230. Concentrations of Na^+, Mg^{2+}, K^+, and Ca^{2+} were not significantly different for controls and treatments, even in treatments in which Ca^{2+} was added. This is contrary to short-term studies that indicate that exposure to 80 μg/L dissolved Cu for 96 h significantly reduced whole body concentrations of Na^+, K^+ and Mg^{2+} by 25%, 30%, and 50%, respectively, in 30-day old *P. paludosa* (Hoang, personal communication).

In freshwater invertebrates, acute Cu exposure has been shown to produce secretions that change the physical properties of the external membrane and alter the permeability of the epithelium, which disrupts homeostasis of osmoregulatory ions between the plasma and the external environment (Cheng 1979). Cheng and Sullivan (1977) found that exposure to 60 μg/L Cu for 36 h resulted in significantly reduced osmolality of hemolymph in the freshwater pulmonate *Biomphalaria glabrata*. The current study indicates that although Cu accumulated in the shell, tissue, and viscera during the 230-day exposure, mineral concentrations were not significantly different, indicating that snails might have acclimated to low Cu exposures and restored their ionic balance. Acclimation to ionic disruption has been documented for other organisms exposed to Cu at sublethal concentrations. Lauren and McDonald (1987) found that *Salmo gairdneri* (steelhead trout) exposed to sublethal waterborne Cu (55 μg/L) for 28 days initially had 55% inhibition of Na^+ uptake and whole body Na^+ decreased by 12.5%, but at the end of the 28 days, both whole body Na^+ and Na^+ uptake returned to control values.

Growth

Growth was measured at two time points during the study. All growth measurements are reported in Table 2. Snails were measured on day 60 because previous studies have indicated that *P. paludosa* enter a phase of rapid growth between 2 and 4 weeks old, which can last several weeks (Hanning 1979; Martin and Doebel 1973). The total length was significantly higher in the control than T2 ($p < 0.02$). The control was also significantly higher than T2 for aperture length ($p < 0.034$), diameter ($p < 0.013$), and wet weight ($p < 0.042$).

Growth measurements were also made on day 230 for all organisms in every replicate, prior to separation for mating. Whereas these measurements, as well as aperture length, diameter, and wet weight, follow a similar trend as day 60 growth measurement, there was no significant difference between growth in control and either treatment.

Table 2 Growth of adult apple snails after 60 and 230 days of aqueous Cu exposure

Treatment	Day[a]	Total length[b] (mm)	Growth rate[b] (mm total length/week)	Aperture length[b] (mm)	Diameter[b] (mm)	Wet weight[b] (g)
Initial	0	4.2 (0.3)	NA[c]	3.9 (0.2)	4.3 (0.4)	0.03 (0.005)
Control	60	22.9 (1.8)	2.18	18.3 (1.1)	20.8 (1.4)	3.6 (0.7)
T1	60	21.1 (1.4)	1.97	17.0 (1.3)	19.1 (1.2)	2.9 (0.6)
T2	60	18.7 (1.8)*	1.69*	15.5 (1.3)*	16.9 (1.7)*	2.3 (0.6)*
Control	230	31.0 (5.4)	0.82	24.5 (3.9)	29.1 (4.9)	9.4 (4.4)
T1	230	30.8 (4.5)	0.81	23.6 (3.3)	30.4 (5.3)	8.8 (4.4)
T2	230	30.4 (5.2)	0.80	24.2 (3.9)	28.5 (5.7)	9.0 (4.6)

[a] $n = 10$ for Initial, $n = 3$ snails per replicate for day 60, $n = 15$ snails per replicate for day 230

[b] Data are mean (standard deviation)

* Significantly different from control ($p < 0.05$)

These data indicate that although exposure to Cu initially decreased growth in both Cu treatments, snails in Cu treatments eventually reached sizes similar to control snails after 230 days. Several studies have determined that exposure to Cu can have adverse effects on vertebrates (e.g., bluegill sunfish) and invertebrates (e.g., *D. magna*), including the Florida apple snail (Hoang et al. 2008a); however, exposure route and duration can significantly affect the results of growth studies. Peña and Pocsidio (2007) found that prereproductive Golden apple snails (*Pomacea canaliculata*) displayed significantly reduced growth during the first 20 days when exposed to high Cu concentrations in water (up to 67.5 μg/L), but by day 50, there was no discernable difference in shell length or growth rates. Pena and Pocsidio (2007) indicated that this phenomenon might be due to redirection of energy from growth to reproduction, as previous studies showed that *P. canaliculata* exhibited decreased growth rates during the breeding season. Marr et al. (1996) stated that factors, such as acclimation, feeding efficiency and behaviors (feeding activity level, appetite suppression), feed conversion, diet composition and exposure regime, might be responsible for initial growth reductions followed by partial or complete recovery of growth rates.

Reproduction

Although the mean time until onset of egg laying for each treatment was similar, it was highly variable within replicates for each treatment (Fig. 2a). T1 had one replicate with the earliest time until onset of egg production (day 140) and the latest (day 208). DeSchamphelaere et al. (2007) reported that for *D. magna* exposed to Cu via diet, time to first brood was unaffected, but the second and third broods were significantly delayed.

The mean cumulative clutch production (or the mean of the total number of clutches produced during the 30-day comparative reproduction period for each treatment) was significantly reduced in the two Cu treatments (8–16 μg/L) by about 30% compared to the control ($p < 0.032$) (Fig. 2b). These results are similar to findings by DeSchamphelaere et al. (2007) for *D. magna*, in which brood production was reduced by 25% by a dietary Cu exposure. Real et al. (2003) found that sublethal dietary Cu exposure to the freshwater snail *Stagnicola vulnerata* significantly reduced the production of egg clutches by 50%. Results of cumulative clutch production are particularly important because 90% of the measurements of Cu concentrations in surface freshwaters in St. Lucie and Martin counties in south Florida were 7.6 μg/L and 14.0 μg/L, respectively (Schuler et al. 2008).

The mean cumulative clutch production was transformed into weekly clutch production. The mean weekly clutch production per reproductive female was 1.8 ± 0.4, 1.2 ± 0.3, and 1.2 ± 0.1 clutches for the control, T1, and T2, respectively. These values are higher than reported by Hanning (1979) for *P. paludosa* in laboratory experiments (0.45–0.56 clutches per reproductive female per week) and field experiments (0.90–0.97 clutches per reproductive female per week) for April and June.

Exposure to Cu did not affect the number of eggs per clutch during the 30-day comparative reproductive period (Fig. 2c). These data are similar to those reported for *P. paludosa* by Hanning (1979) in Florida for Monkey Box Bay (28.3 eggs per clutch), Loxahatchee Wildlife Refuge (28.5 eggs per clutch), the St. Johns River Impoundment (26.2 eggs per clutch), and Lake Woodruff National Wildlife Refuge (28.3 eggs per clutch) between 1973 and 1979. Pena and Pocsidio (2007) also reported that Cu exposure (up to 67.5 μg/L) had no effect on the number of

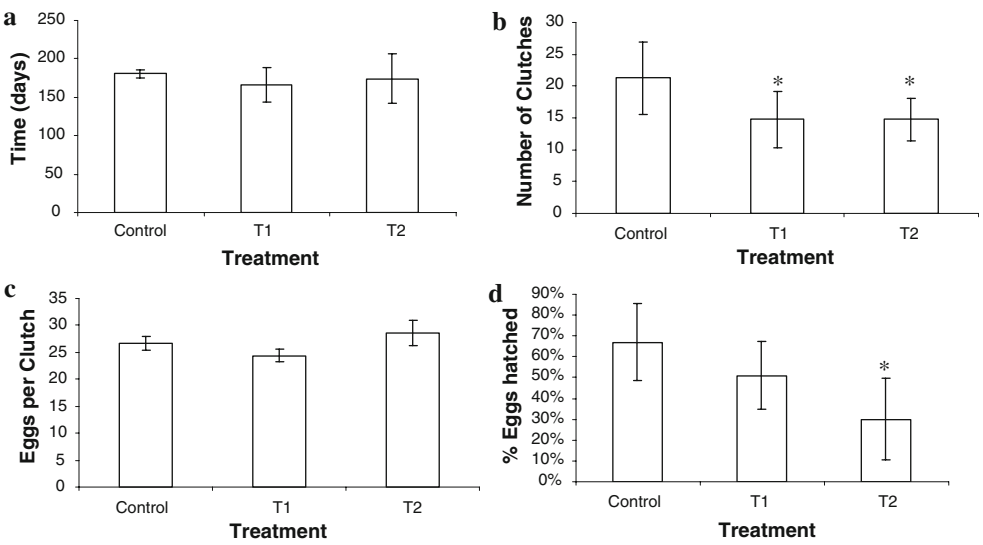

Fig. 2 Reproductive end points in adult apple snails: (**a**) days to first clutch production, (**b**) cumulative clutch production, (**c**) number of eggs per clutch, (**d**) hatching percentage. Data are mean of four replicates; error bars are standard deviations. * Indicates significant difference from control ($p < 0.05$)

eggs per clutch in *P. canaliculata*, but both DeSchamphelaere et al. (2007) and Real et al. (2003) reported that exposure to dietary Cu significantly reduced brood size or number of eggs per clutch in *D. magna* and *S. vulnerata,* respectively.

Egg hatching was recorded for clutches produced during the 30-day comparative reproductive period (Fig. 2d). Clutches that had 0% hatching or that were laid on the walls of the chambers were not included in this analysis. Hatching was significantly reduced in the T2 treatment versus the control ($p < 0.008$) but not in the T1. Hanning (1979) reported that for *P. paludosa,* mean hatching in the field (with ambient air temperature) was 76.6% (29–40°C) and 86.8% (30–35°C). In the laboratory, hatching was reduced to 64.3% at 25–27°C, indicating a relationship between air temperature and hatching. De Lara (1988) found that control *P. canaliculata* displayed 75% hatching under laboratory conditions. Laboratory hatching (64.3%) from Hanning (1979) is similar to hatching in the control in the present study (67%), which was also held at 26–27°C. Real et al. (2003) found that dietary Cu exposure reduced hatching from 89% to 42% in *S. vulnerata* exposed to Cu versus controls. Pena and Pocsidio (2007) reported that Cu concentrations in water (up to 67.5 µg/L) had no effect on hatching in *P. canaliculata.*

Although not all reproductive end points for *P. paludosa* were affected by chronic, sublethal Cu exposures, clutch production and hatching were significantly reduced in this study. DeSchamphelaere (2007) suggested that possible mechanisms for Cu-induced inhibition of reproduction in *D. magna* might be increased metabolic cost, reduced energy acquisition, or targeted inhibition of reproduction.

Whole Body Copper Concentrations and Early Growth in F_1 Generation

The mean whole body Cu concentrations for newly hatched F_1 snails (<48-h-old, $n = 10$ per replicate) from the first clutches produced in each replicate were 33.6 ± 6.8, 54.5 ± 11.0, and 42.3 ± 8.7 mg/kg Cu dw in the control, T1, and T2, respectively (Table 3). Treatments did not differ significantly ($p < 0.10$), but there appears to be a slight increase in whole body Cu in the Cu treatments. This trend might suggest that when exposed to high levels of Cu, maternal transfer might occur. However, newly hatched F_1 snails (<48-h-old, $n = 10$ per replicate) from the first clutches produced after adults were thinned for the comparative reproductive study (day 230) had mean whole body Cu concentrations of 36.0 ± 4.6, 39.9 ± 5.2, and 44.6 ± 11.5 mg/kg Cu dw in the control, T1, and T2, respectively. No significant differences in Na^+, Ca^{2+}, K^+, or Mg^{2+} concentrations were observed between newly hatched F_1 snails in control and treatments for either collection.

Maternal transfer of Cu has not been well researched; however, data suggest that other metals (i.e., Hg, Cd, Se) can pass from parent to offspring as a means of elimination (Lam and Wang 2006; Tsui and Wang 2004). Tsui and Wang (2004) reported that in *D. magna*, maternal transfer of Hg and MeHg was an important pathway for elimination for the mother. Lam and Wang (2006) found that in *D. magna* fed Se, 19–24% of Se transferred to the F_1 generation, but maternal transfer efficiency decreased from F_1 to F_2. From the current study, it appears that Cu did not transfer from F_0 to F_1 snails, or only negligable amounts of Cu transferred from F_0 to F_1 apple snails, indicating that maternal transfer is not a major elimination route for Cu in the Florida apple snail.

Both F_1 survival and growth did not differ between the control and treatments (Table 3). Twenty-eight-day growth measurements for F_1 in the present study were less than reported by Hoang et al. (2008a) for the Florida apple snail. Snails in the present study were held under static renewal conditions in water-only exposures, whereas snails in the previous study were held under flow-through conditions with soil/sediment that might have provided additional organic matter or minerals for the snail. Bossuyt and Janssen (2003) reported that acclimation of *D. magna* to Cu altered natural tolerance and was passed on to subsequent generations, a phenomenon that did not occur in the present study. Maternal exposure to aqueous Cu did not have advantageous or deleterous effects on survival or growth of F_1 apple snails.

Table 3 Survival, growth, and whole body Cu concentrations of juvenile F_1 apple snails

Treatment	Day[a]	Survival[b] (%)	Total length[b] (mm)	Growth rate[b] (mm total length/week)	Aperture length[b] (mm)	Diameter[b] (mm)	Whole body Cu concentration[b] (mg/kg dw)
Control	28	90.0 (8.2)	10.40 (0.2)	1.60	9.06 (0.2)	9.60 (0.2)	33.6 (6.8)
T1	28	90.0 (14.1)	10.00 (0.6)	1.50	8.65 (0.6)	9.22 (0.6)	54.5 (11.0)
T2	28	85.0 (1.0)	10.22 (0.6)	1.55	8.90 (0.4)	9.56 (0.5)	42.3 (8.7)

[a] $n = 10$ at beginning of growth study

[b] Data are mean (standard deviation)

Conclusions and Implications

Florida apple snails exhibit significantly reduced clutch production (8–16 μg/L) and egg hatching (16 μg/L) as a result of Cu exposures. Snails also accumulated Cu from water with elevated concentrations (8–16 μg/L). These findings gain in importance because Cu concentrations used in this study are similar to the Cu concentrations in surface freshwaters in south Florida (e.g., St. Lucie and Martin counties).

Copper concentrations in water in the present study shown to produce significant biological effects in the Florida apple snail are also significantly lower than those Cu concentrations being produced in overlying water (308.2 μg/L) after flooding agricultural soils from south Florida lands acquired under the CERP. Given that agricultural land acquired under the CERP will become inundated and eventually inhabited, many species might accumulate Cu and ultimately become adversely affected. The Florida apple snail and other species of importance in freshwater food chains of south Florida might be at risk, including their predators.

Additional research is required to assess the repercussion of chronic Cu exposure on the population dynamics of the Florida apple snail and whether accumulation of Cu in the tissue of the apple snail transfers to their predators.

Acknowledgments We thank Robert Frakes of the US Fish and Wildlife Service, Pamela Bachman (FIU), and Kathy Moore (Clemson University) for their assistance with completing this study. We thank Philip Darby of the University of West Florida for technical support on the Florida apple snail. This is SERC contribution No. 403.

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