SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 32, No. 10, pp. xx–xx, 2013
© 2013 SETAC
Printed in the USA

Environmental Toxicology

IMPOSEX AND NOVEL MECHANISMS OF REPRODUCTIVE FAILURE INDUCED BY TRIBUTYLTIN (TBT) IN THE FRESHWATER SNAIL *POMACEA CANALICULATA*

MAXIMILIANO GIRAUD-BILLOUD,*†‡ ISRAEL A. VEGA,†‡§ RODOLFO G. WUILLOUD,§ MARÍA E. CLÉMENT,† and ALFREDO CASTRO-VAZQUEZ†‡§

†Institute of Physiology, School of Medicine, National University of Cuyo, Mendoza, Argentina
‡Institute of Histology and Embryology, National Research Council, Mendoza, Argentina
§Institute of Basic Sciences, National University of Cuyo, Mendoza, Argentina

(Submitted 26 December 2012; Returned for Revision 28 January 2013; Accepted 12 June 2013)

Abstract: The effects of tributyltin (TBT) on mortality and reproduction were studied in the invasive snail *Pomacea canaliculata*. The nominal median lethal concentration (LC50) was 9 μg TBT/L, after 28 d. The nominal no-observed effect on lethality concentration (NOEC_L) was 6 μg TBT/L after the same period. Male–female couples and females that had been group-mated but were isolated from males during the experiment (isolated females) were exposed (for 28 d) to either 0 μg/L or 6 μg/L of TBT (nominal NOEC_L-exposed). Copulation and oviposition frequencies, egg clutch mass, and percentage of egg fertility were recorded. Gonads (both sexes) and the seminal receptacle (females) were studied histologically at the end of the experiment. A significant decrease in copulation frequency was observed in mated-exposed females. Exposure also decreased oviposition frequency of mated-exposed and isolated-exposed females, but only the latter reached significance. No differences in either egg clutch mass or percentage of fertility were observed at first oviposition, but both parameters were drastically reduced in subsequent egg clutches of exposed females. No histological alterations were observed in gonads of TBT-exposed animals; however, sperm storage in the seminal receptacle was drastically decreased in exposed females. Imposex but no oviductal obstruction was observed in all exposed females. It is concluded that TBT induces reproductive failure in *P. canaliculata* by decreasing copulation frequency and by severely affecting sperm storage by the female. *Environ Toxicol Chem* 2013;32:xx–xx. © 2013 SETAC

Keywords: Endocrine disruptors Fertilization Spermicides Imposex Apple snails

INTRODUCTION

An endocrine disruptor (ED) is an exogenous agent that interferes with a particular hormonal system and consequently could affect the maintenance of homeostasis, reproduction, development, and/or behavior [1]. Endocrine disruption in several vertebrates and invertebrates has been the center of interest of numerous reports on the adverse effects of pesticides and industrial chemicals [2,3]. The best known example of endocrine disruption is the development of imposex (superimposition of male sexual characteristics onto females [4]) observed in gastropods that have been exposed to organotin EDs such as tributyltin (TBT) or triphenyltin (TPT; for review see Oehlmann et al. [5] and Titley-O'Neal et al. [6]).

Tributyltin is a biocide that has been extensively used in ship antifouling paints. Moreover, organotins have been used in different commercial applications such as polyvinyl chloride stabilizers, fungicides, molluscides, rodent repellants, and wood preservatives [7,8]. Although the use of TBT was restricted during the 1990s, high levels are still found in aquatic ecosystems mainly because all usages have not been banned [9,10]. Furthermore, several regulations on the use of TBT are currently being enforced in South American countries, but extensive contamination of marine environments (particularly on the Atlantic coast) has been reported [11,12]. Unfortunately, similar studies in freshwater environments are lacking. Low concentrations of this ED induce imposex, but in severely affected cases, reproductive failure has been reported in gastropods, either because the growth of male-like genitalia blocks the oviduct or because of spermatogenesis in the ovary [13–20].

The apple snail *Pomacea canaliculata* (Caenogastropoda, Ampullariidae) is a native species of the LaPlata River basin (South America), but has invaded freshwater habitats of Southeast Asia, North America, and Europe [21–24]. The abundance of this invasive snail in different habitats, and their ability to tolerate harsh environmental conditions [25,26] ensures its invasive capability [21] and may allow its use as a bioindicator of water pollution [27].

Two morpho-functional aspects of *P. canaliculata* are important to understand the findings we will report here. First, females of these species (as well as those of all other studied Ampullariidae) show a rudimentary copulatory apparatus that may grow after reaching sexual maturity [28,29]. The growth of this rudiment, because of its position relative to the oviduct [28], is unable to interfere with oviposition, a condition that differs substantially from those found in other caenogastropods and vetigastropods [6,30]. Second, oviposition depends on the existence of fertilized eggs in the female tract of *P. canaliculata* [31,32].

We first made a range-finding determination of the lethal effects of nominal concentrations of TBT, so as to work with sublethal concentrations to determine the effect/s of TBT on reproductive mechanisms.

MATERIALS AND METHODS

Animals

Sexually mature males and females (4 mo old at the beginning of the experiment) from a cultured strain of *P. canaliculata*

---

* Address correspondence to mgiraudbilloud@gmail.com.

DOI: 10.1002/etc.2310

were used. The original stock was collected at Rosedal Lake (Palermo, Buenos Aires, Argentina) and voucher (alcohol-preserved) specimens of the original population and of the cultured strain were deposited with the collection of Museo Argentino de Ciencias Naturales (Buenos Aires, Argentina; lots MACN-In 35707 and MACN-In 36046, respectively). The culture conditions were: 1) room temperature 23 °C to 26 °C, 2) room relative humidity approximately 80%, 3) artificial lighting 14 h/d, 4) water used for culture was tap water and was changed thrice weekly, and 5) the animals were fed ad libitum with lettuce from Monday through Friday, supplemented with fish food pellets (Peishe Car Shulet) on Thursday and with excess toilet paper (Higienol for Export) on Friday. The latter provides cellulose as a nutrient and does not spoil the water during weekends, as excess lettuce would do. Sex was determined externally by the shape of the operculum [33,34]. Experiments were run in 23.5 cm × 19.5 cm × 32 cm aquaria, where the experimental solutions were kept 5 cm deep, and only lettuce and fish food pellets were used during the reported experiments, which needed daily surveillance, even during weekends.

Experimental sets

Concentration- and time-dependent lethal effects of TBT. Lethality induced by TBT was determined for snails exposed to the following nominal concentrations: 0 μg/L, 3 μg/L, 6 μg/L, 12 μg/L, 24 μg/L, or 50 μg/L of tributyltin chloride (Sigma-Aldrich, T50202). The commercial TBT solution was first mixed with 70% ethanol (0.5–8 mL in 399.5–392 mL), and then 15 μL of these intermediate solutions were added to tap water to obtain the different TBT nominal concentrations used. Also, ethanol was added to tap water to obtain a final 0.001% concentration in control groups. Three replicate groups of 4 snails (2 males and 2 females) were allotted to each aquarium with the different nominal TBT concentrations. The solutions were changed 3 times weekly. Snail deaths were recorded after 14-d and 28-d exposure. The no-observed-lethal-effect concentration ($NOEC_L$) and the median lethal concentration (LC50) were estimated with Prism version 5.0 (GraphPad Software) using the nonline fit of data tool and the log (agonist) versus response curve.

Tin determinations in tissues. This was made using inductively coupled plasma-mass spectrometry (ICP-MS) as an indirect approach to exposure verification. Laboratory ware for this purpose was washed in HCl for 1 wk (17.5%, v/v) and subsequently with HNO_3 (32.5%, v/v) for another week. The remaining acid was washed out with 3 passages in abundant type I water (ASTM International) and then oven-dried at 60 °C and stored until use.

Two groups of 9 females were exposed to either 6 μg/L of TBT (nominal $NOEC_L$) or to tap water with 0.001% ethanol for 28 d. Each aquarium (20 cm × 38 cm × 52 cm) was divided into 6 equal communicating compartments (1 compartment was left empty in each aquarium). The cephalopodal and visceral masses (the latter containing the mantle cavity and all reproductive organs) were separated and homogenized in type I water (ASTM International), lyophilized, and milled. One-gram samples of pooled cephalopodal and visceral masses were added to 10 mL of concentrated HNO_3 and 5 mL of 30% H_2O_2 and left overnight at room temperature. Afterwards, each mixture was boiled on a hot plate for 2 h; after cooling, the completely clear digests were diluted with 50 mL of ultrapure water (18 MΩ cm) prior to ICP-MS determinations of total tin, using an ELAN DRC-e instrument (PerkinElmer SCIEX). Sample introduction into the plasma was made with a Meinhard concentric nebulizer with a quartz cyclonic spray chamber at 4 °C. The operation of the ICP-MS instrument was optimized using a multielemental tuning standard solution containing 10 μg/L tin. The instrumental conditions used were as follows: inductively coupled plasma-radio frequency power, 1500 W; plasma gas flow, 15 L/min; auxiliary gas flow, 1.2 L/min; nebulizer gas flow, 0.81 L/min; skimmer and sampler cones type, nickel; and detector mode, dual. The abundances of ^{118}Sn and ^{120}Sn are the highest, and both isotopes are little interfered with by oxides, polyatomic ions, and hydride, but ^{118}Sn was selected for analysis, as at this mass-to-charge (m/z) ratio, the Sn signal was less disturbed than ^{120}Sn by chloride and argon. Analytical standards with different tin concentrations were prepared by serial dilution of a 1 mg/L tin mother standard solution in ultrapure water. External calibration was used for quantification of tin, and blanks were prepared with the same reagents and quantities employed for sample preparation. Results were expressed as concentration (μg/g dry organ mass) and load (μg) of tin in both organ masses on a per snail basis.

Effect of TBT-$NOEC_L$ on reproductive parameters

Snails were allotted to 1 of 2 groups: 1) 4 aquaria containing 5 male–female couples, and 2) 4 aquaria containing 5 mature females only (but that had been group-mated before with mature males). Then, half of the animals in each group were exposed to the nominal $NOEC_L$ (6 μg TBT/L), while unexposed snails were used as controls. Each aquarium (20 cm × 38 cm × 52 cm) was divided into equal communicating compartments, as in the preceding experiment. The animals were fed ad libitum with lettuce every day of the week during the experiment, and the diet was supplemented with fish food pellets (Peishe Car Shulet) on Tuesday and Thursday.

The frequencies of copulation and oviposition were recorded twice daily (at 8:00 AM and 1:00 PM, because more than 80% of copulations and all ovipositions are recorded under this schedule [35]; also, the mass of each egg clutch was determined at 8:00 AM. The percentage of fertile eggs was determined in each clutch 1 d to 2 d before the expected hatching [36].

The animals were sacrificed at the end of the experiments, and gonadal samples from both sexes and the whole seminal receptacles from females were excised out and fixed in dilute Bouin's fluid. The fixed tissues were subsequently dehydrated in an ethanol series and embedded in a resin-paraffin mixture (Histoplast, resin 50%/paraffin 50%). Sample sections were obtained from gonads, but the seminal receptacles were semiserially sectioned. Subsequently, the sections were stained with Harris hematoxylin–eosin. Digital light micrographs were obtained with an AxioCam HRc mounted on a Zeiss Imager Z.1 microscope. Also, the rudimentary copulatory apparatus and surrounding structures were dissected out, fixed in formalin, dehydrated in an ethanol/acetone series, critical point dried, and gold-coated for examination under a LEO 1450VP scanning electron microscope.

Statistical analyses

Differences between 2 groups were analyzed with Student's *t* test (copulation frequency). For multigroup comparisons, the Gaussian distribution of variables and the homogeneity of variances were evaluated by D'Agostino and Pearson's and Bartlett's tests, respectively (Prism 5.0, 2007; GraphPad Software). Only the egg clutch mass data met the criteria (after square root transformation) to be evaluated with parametric one-way analysis of variance (ANOVA) and the Tukey test for post hoc analysis. Other multigroup comparisons were made with the nonparametric Kruskal–Wallis one-way ANOVA and Dunn's test (Prism 5.0, 2007). Significance level was fixed at $p < 0.05$.

RESULTS AND DISCUSSION

The ability of organotin compounds to induce imposex has been reported in more than 250 species of gastropods [6]. Nevertheless, the deleterious effects of TBT on aspects of reproduction other than imposex have received lesser attention in gastropods, although they have been reported to involve oviductal blockade, ovarian dysmaturation, and ovotestis development [13,16,18–20,37,38]. In the present study we have observed deleterious effects of TBT on both copulation and oviposition frequency, and on sperm storage in the seminal receptacle, which are novel mechanisms for TBT-induced reproductive failure in a gastropod. In addition, TBT-induced development of imposex was also established for this species.

When lethal toxicity of TBT on *P. canaliculata* was explored, the nominal NOEC_L found was either 9 μg/L or 6 μg/L, after 14-d or 28-d exposure, respectively, while the LC50 was either 12.9 μg/L or 9.0 μg/L after the same periods (nominal concentrations; Figure 1). However, potential uncertainties in the actual exposure concentrations exist, and hence the nominal effect concentrations reported should not be regarded as quantitative benchmarks for risk assessment purposes.

Despite this, tin was determined in soft tissues of control animals and of animals exposed for 28 d to the nominal NOEC_L (6 μg TBT/L; Table 1) as an indirect means of confirming actual TBT exposure. Tin was found in tissues of control snails, which is not surprising in view of the widespread environmental presence of this metal [39–41], generally considered nontoxic in its inorganic form [8]. However, the tin load was not appreciably increased in the cephalopodal mass of TBT-exposed snails but was approximately 4 times higher in the visceral mass of exposed animals, which includes the reproductive organs (Table 1). Also, it is worth mentioning that Wang et al. [42] found a significantly higher accumulation of TBT in reproductive organs, compared with the remaining tissues, in the neogastropod *Thais clavigera*.

Exposure of couples of *P. canaliculata* to the nominal NOEC_L (6 μg TBT/L) during 28 d resulted in a marked and statistically significant decrease in copulation frequency (Figure 2A; Student's *t* test). Also, the oviposition frequency was diminished by TBT exposure in both mated and isolated females, while the difference was statistically significant only for isolated females (Figure 2B; one-way ANOVA, followed by Tukey's post hoc test, $p < 0.05$). Copulation and oviposition are behavioral sequences whose execution depends (among other factors) on the activation of neural networks that may be affected by the neurotoxic action of TBT [43,44]. In addition, the smaller effect on mated females should be due to the already mentioned fact that oviposition depends on the existence of fertilized eggs in this species [31,32] and to the persistence of some copulatory activity and possible sperm transfer to the mated-exposed couples.

In general, the egg clutch mass was higher in control than in exposed females, and this was more evident in isolated than in mated females, because no additional sperm transfer could occur in the former. However, the first egg clutch showed significant differences only between both control groups and the isolated-exposed group (Figure 2C). Egg clutch masses in mated-exposed females were only significantly different from controls when the second and the last clutches laid were compared (Figure 2C); also, the last clutches differed significantly from the first one in these females. Similarly, other studies have shown a decrease in the oviposition frequency and in the number of eggs per clutch in organotin-exposed *M. cornuarietis* [45,46] and *Biomphalaria glabrata* [47].

Moreover, fertility of eggs was severely affected in TBT-exposed isolated females, but only after the first egg clutch was laid during exposure (Figure 2D), which may also be related to remaining sperm in the seminal receptacle at the beginning of the experiment. The majority of eggs were infertile from the second egg clutch laid by exposed females, while egg clutches from control females (either mated or isolated) contained approximately 80% fertile eggs during the entire experimental period (Figure 2D).

Environmental organotin exposure has been reported to induce ovotestis development and spermatogenesis in female vetigastropods [16,19] as well as in some [18,20] but not all [13] neogastropods. Oocyte dysmaturation (atresia) associated with a high body burden of TBT (in the μg/kg range) has also been reported in a littorinimorph gastropod collected near harbors [37]. However, the concentration of 6 μg/L did not produce any morphological alteration of male or female gonads in *P. canaliculata*, an architaenioglossan caenogastropod

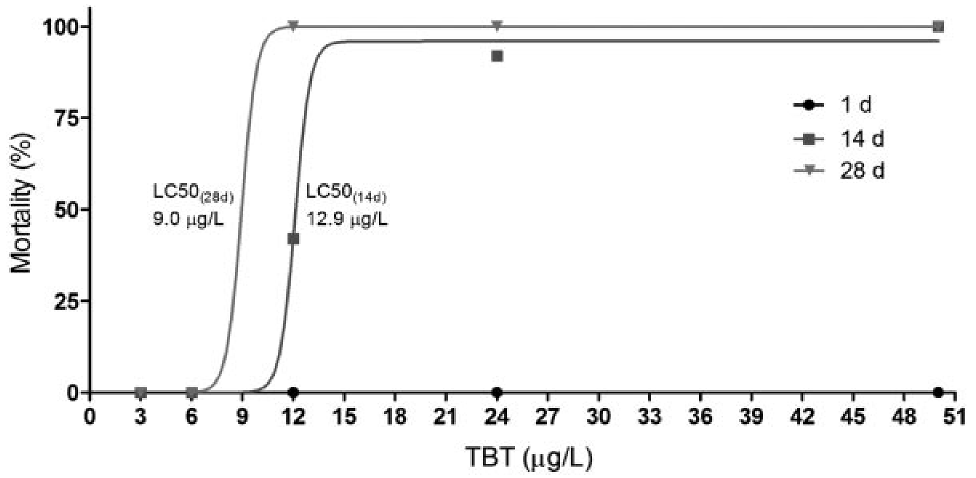

Figure 1. Time- and concentration-dependent mortality of adult *Pomacea canaliculata* exposed to tributyltin (TBT) ($n = 12$ per group). LC50 = median lethal concentration.

Table 1. Tin concentration in the cephalopodal and visceral mass after tributyltin (TBT) exposure (pools from 9 animals)

	Organ dry mass (g/snail, mean ± SEM)	Tin concentration (µg/g dry mass)	Tin load (µg/organ dry mass/snail)
Cephalopodal mass (control)	0.320 ± 0.019	24.7	7.91
Visceral mass (control)	1.101 ± 0.111	29.3	15.59
Cephalopodal mass (exposed)	0.323 ± 0.020	24.4	7.89
Visceral mass (exposed)	1.374 ± 0.188	91.9	61.36

SEM = standard error of the mean.

(Figure 3), emphasizing that different mechanisms may be employed by organotins to interfere with reproduction in the wide diversity of the class Gastropoda.

Because of the already mentioned relationship between oviposition and the availability of fertilized eggs in the genital tract, we examined the seminal receptacle in both control and exposed females (either mated or isolated), because the seminal receptacle is the site where oocytes are received through the visceral oviduct and where sperm are stored, while retaining their fertilizing capacity for months after copulation [48]. All these findings, together with direct sperm transfer to the seminal receptacle [49], indicate that this is the site of fertilization in *P. canaliculata*.

Most stored sperm were aligned as palisades in the branched pouches of the seminal receptacle of control females, even though disorganized sperm masses may also be seen in the larger ducts in mated-control females (Figure 4A) and may represent recently deposited sperm. Also, some empty pouches may also be seen in control females, particularly in those that were isolated (Figure 4B). The TBT-exposed females (either mated or isolated) showed some pouches containing only small groups of palisade-aligned sperm (Figure 4C) or pouches that were depleted of stored sperm (Figure 4D). In particular, the decreased number of sperm-loaded pouches in the isolated-exposed group (compared with the mated-exposed group) indicates that TBT has a direct deleterious effect on sperm

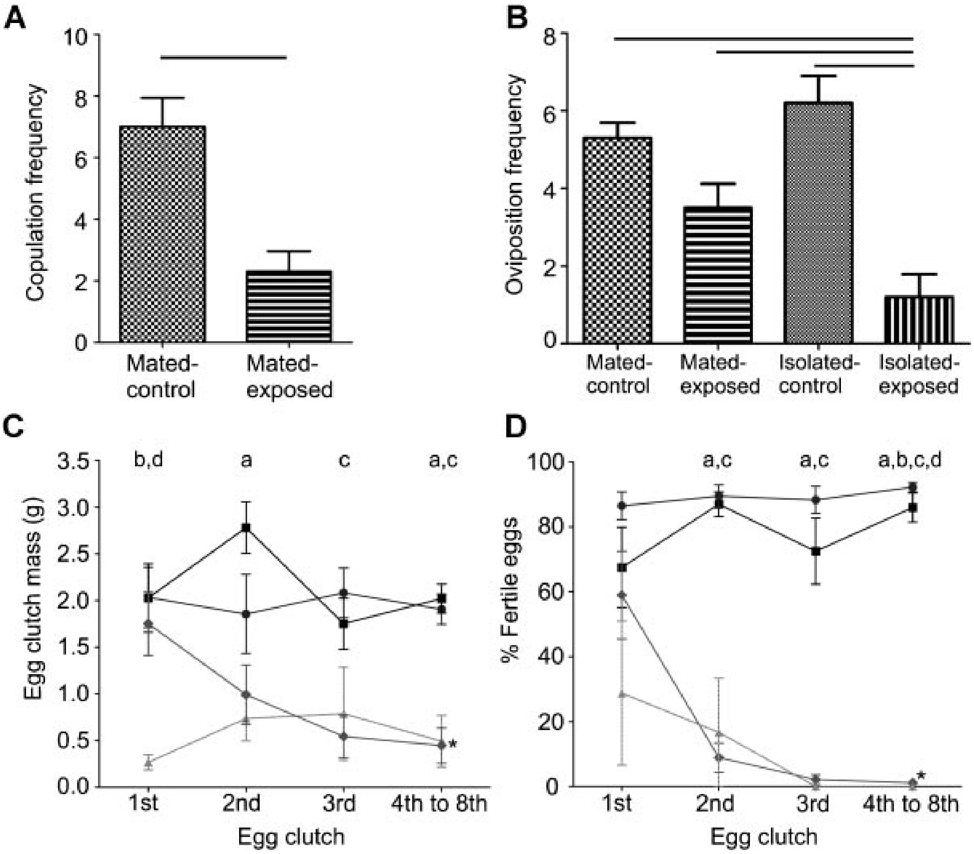

Figure 2. Effect of exposure for 28 d to 6 µg/L of tributyltin (TBT, nominal no-observed effect on lethality concentration [$\mathrm{NOEC_L}$]) on copulation by male-female couples and on oviposition by both mated and isolated females ($n = 10$ per group). **(A)** Decrease in copulation frequency in couples that were exposed or not to TBT (episodes/mo, mean ± standard error of the mean [SEM]; Student's *t* test). **(B)** Oviposition frequency in mated-control females, mated-exposed females, isolated-control females, exposed-isolated females (episodes/mo, mean ± SEM; one way analysis of variance/Tuckey). **(C)** Egg clutch mass of the first, second, third and fourth to eighth egg clutches laid by each female during the experimental period in mated-control females (■), mated-exposed females (◆), isolated-control females (•), exposed-isolated females (▲) (g, mean ± SEM; Kruskal-Wallis/Dunn). **(D)** Percent of fertile eggs in each clutch (the groups are identified as indicated in **(C)**; mean ± SEM; Kruskal-Wallis/Dunn). Statistically significant differences between groups in panels **(A)** and **(B)** are indicated with horizontal lines. Significant differences in panels **(C)** and **(D)** (within the same egg clutch) are indicated by superscripts: a = mated-control versus mated-exposed groups; b = mated-control versus isolated-exposed, c = mated-exposed versus isolated-control, d = isolated-control versus isolated-exposed. The asterisk in panels **(C)** and **(D)** indicates significant differences between the first and fourth to eighth egg clutches in mated-exposed females.

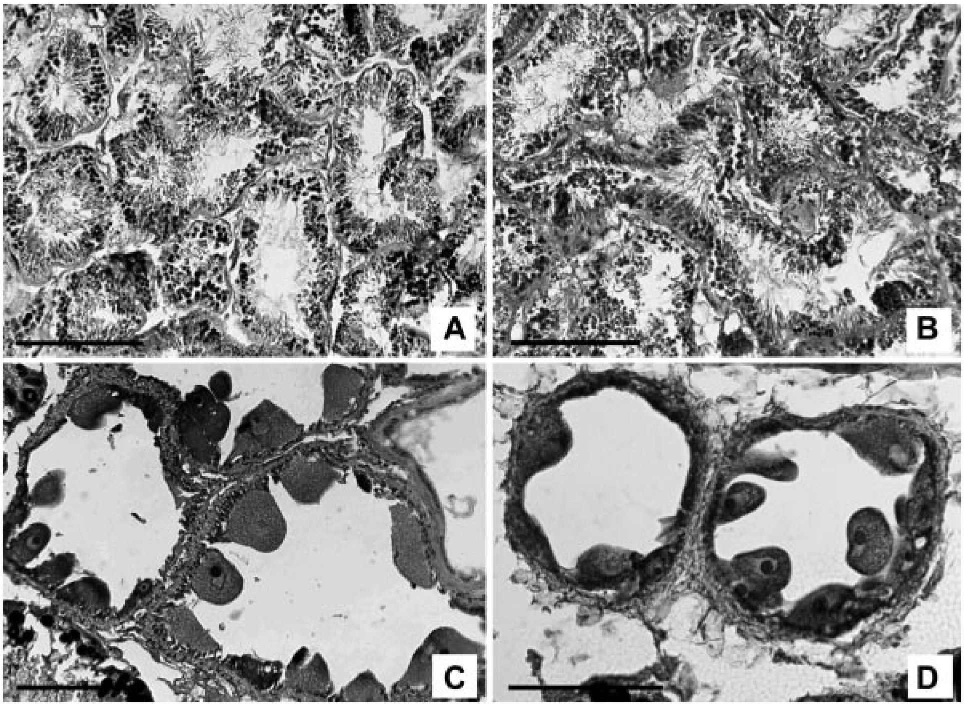

Figure 3. Micrographs of male and female gonads from control and exposed animals (tributyltin [TBT], nominal 6 μg/L). (**A, B**) Spermatogenesis in control and exposed males, respectively: testis tubules show numerous tails of mature sperm in both groups. (**C, D**) Oogenesis in control and exposed isolated females, respectively; ovarian tubules show small and large oocytes (histology was similar in mated females). Scale bars = 100 μm.

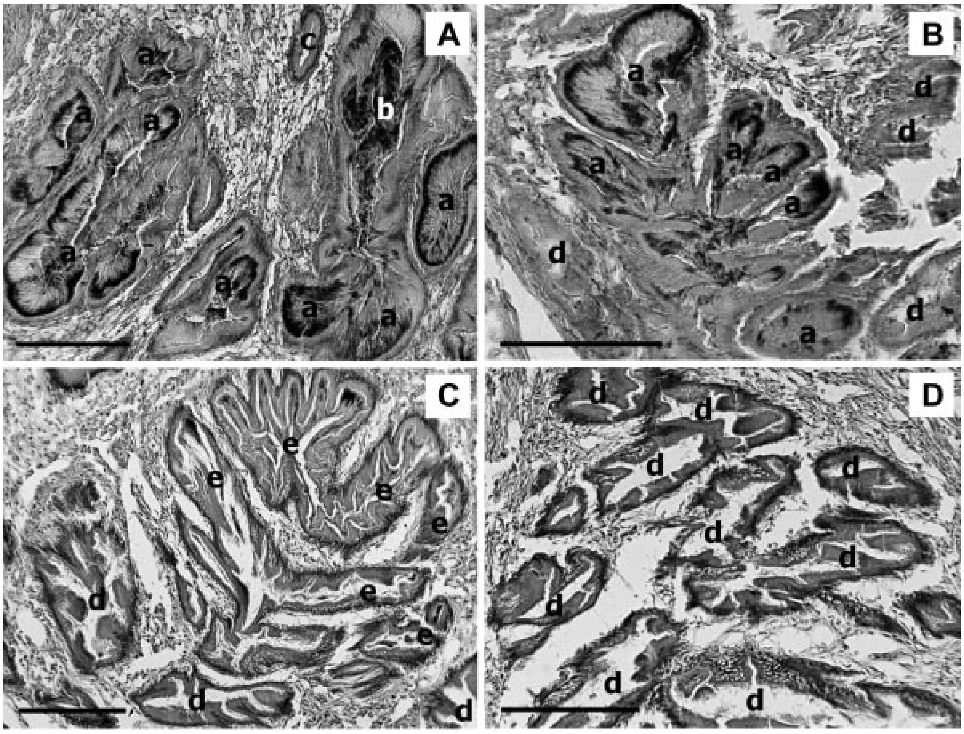

Figure 4. Micrographs showing the sperm pouches of the seminal receptacle. (**A**) Mated-control female. (**B**) Isolated-control female. (**C**) Mated-exposed female. (**D**) Isolated-exposed female. a = pouches lined by sperm in palisade alignment; b = sperm bulk within the lumen of a pouch already lined by sperm in a palisade alignment; c = oviduct; d = pouches with no sperm; e = pouches with small aligned groups of sperm at the end of some branches. Scale bars = 100 μm.

storage (i.e., not mediated by a diminished copulatory frequency). This direct effect may be correlated with the finding of an increased tin load in the visceral mass (Table 1), which includes the seminal receptacle and the other reproductive organs.

All females exposed to 6 μg/L (nominal $NOEC_L$) in the present study showed an enlarged penial sheath compared with that of unexposed females of the same age (4 mo at the beginning of exposure, Figures 5). A full glandular complement and a pouch with a long penis within were found in all exposed

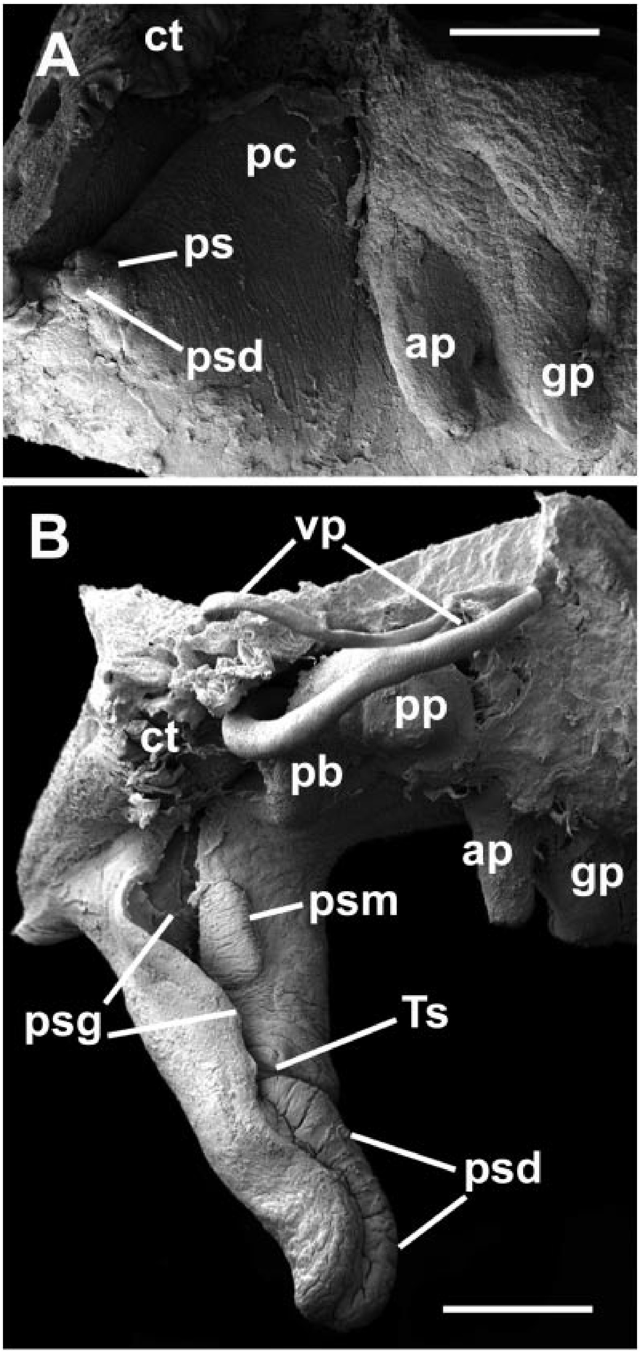

Figure 5. Copulatory rudiments of control (**A**) and tributyltin (TBT)-exposed (**B**; nominal 6 μg/L, 28 d) adult females (5 mo old) of *Pomacea canaliculata*. ap = anal papilla; gp = genital papilla; vp = vermiform penis; pb = penial bulb; pcr = penial complex rudiment; pp = penial pouch; ps = penial sheath; psd = penial sheath distal gland; psg = penial sheath groove; psm = penial sheath medial gland; psr = penial sheath rudiment; Ts = T sulcus. Scale bars = 1 mm.

females in the current experiments (the penis appears stretched out of the penial pouch in Figure 5B). This is the first report in which imposex was induced by TBT in *P. canaliculata* compared with proper controls of the same age (exposed from 4 mo old). Such controls were lacking in a previous report [50], and we have found that age (beyond 4 mo) is associated with further growth of the copulatory apparatus [28,29]. Even though the female rudimentary copulatory apparatus continues to grow in adult unexposed females [28,29] in no case was a degree of development such as that shown here in TBT-exposed females (Figure 5B) observed in those studies. It should be noted, however, that no blockade of the oviduct can occur because of the relative position of the oviduct and the abnormally grown copulatory apparatus, a condition that differs substantially from those found in other caenogastropods and vetigastropods [6,30].

It should also be mentioned that no effects of the 6 μg/L nominal concentration (other than the already mentioned reproductive effects) were observed in the present study. With higher (and lethal) concentrations, however, death was sometimes preceded by a state of hypertonic paralysis, which may reflect the proposed neurotoxic activity of TBT [43,44].

Future studies on the mechanism of the diminishing effect of TBT on copulation and oviposition frequency should also consider this possible neurotoxic activity. On the other hand, we are not aware of any study on the mechanisms of the prolonged maintenance of the fertilizing capacity of sperm in the seminal receptacle of gastropods, which in the case of *P. canaliculata* may last for months [48], so that no hypothesis can be advanced regarding the mechanism of the sperm depletion observed here.

CONCLUSIONS

Our observations on the effect of TBT exposure (6 μg/L, nominal concentration) indicate the primary effect/s of this organotin in reducing reproductive performance in this highly invasive snail *P. canaliculata* is the decrease in available sperm for fertilization, which is achieved by the concurrent deleterious effects on copulation frequency and sperm storage, and which in turns results in decreased oviposition frequency, egg clutch size, and egg fertility.

Also, TBT clearly increased the development of the rudimentary female copulatory organs in adulthood (imposex), which has been shown here for the first time (with adequate controls) in *P. canaliculata*.

Other possible causes of reproductive failure that have been studied in other gastropod species (oviduct blockade, impaired oogenesis) do not occur as a consequence of TBT treatment in *P. canaliculata*.

Acknowledgments—We thank L. Martínez, Universidad Nacional de San Luis (Argentina), for help with the inductively coupled plasma mass spectrometry determinations. The technical help of A.M. Carrión and the criticisms of 2 anonymous reviewers are also acknowledged. The present study was supported by grants from Universidad Nacional de Cuyo, Consejo Nacional de Investigaciones Científicas y Técnicas, and Fondo Nacional de Ciencia y Técnica (FONCyT) of Argentina.

REFERENCES

1. Crisp T, Clegg E, Cooper R, Wood W, Anderson D, Baetcke K, Hoffmann J, Morrow M, Rodier D, Schaeffer J. 1998. Environmental endocrine disruption: An effects assessment and analysis. *Environ Health Perspect* 106:11–56.
2. World Health Organization. 2002. ICPS global assessment of the state of the science of endocrine disruptors. Geneva, Switzerland.
3. Albanis T, Allera A, Bachmann J, Barbaglio A, Berntsson P, Dittmann N, Carnevali D, Ciceri F, Dagnac T, Duft M. 2006. Executive summary on the project results of COMPRENDO–Comparative Research on Endocrine Disrupters. EU project EVK1-CT-2002-0012 9. [cited 2013 June 12]. Available from: http://wwwcomprendoprojectorg.
4. Smith BS. 1971. Sexuality in the American mud snail, *Nassarius obsoletus* Say. *J Mollusc Stud* 39:377–378.
5. Oehlmann J, Di Benedetto P, Tillmann M, Duft M, Oetken M, Schulte-Oehlmann U. 2007. Endocrine disruption in prosobranch molluscs: Evidence and ecological relevance. *Ecotoxicology* 16:29–43.
6. Titley-O'Neal CP, Munkittrick KR, MacDonald BA. 2011. The effects of organotin on female gastropods. *J Environ Monit* 13:2360–2388.
7. Bennett R. 1996. *Industrial Manufacture and Applications of Tributyltin Compounds, Tributyltin*, 1st ed. Cambridge University Press, Cambridge, MA, USA.

8. Hoch M. 2001. Organotin compounds in the environment—An overview. *Appl Geochem* 16:719–743.
9. Antizar-Ladislao B. 2008. Environmental levels, toxicity and human exposure to tributyltin (TBT)-contaminated marine environment: A review. *Environ Int* 34:292–308.
10. Fent K. 2006. Worldwide occurrence of organotins from antifouling paints and effects in the aquatic environment. In Konstantinou I, ed, *Handbook of Environmental Chemistry*, Vol 50. Springer, Berlin, Germany, pp 71–100.
11. Bigatti G, Primost MA, Cledón M, Averbuj A, Theobald N, Gerwinski W, Arntz W, Morriconi E, Penchaszadeh PE. 2009. Biomonitoring of TBT contamination and imposex incidence along 4700 km of Argentinean shoreline (SW Atlantic: from 38S to 54S). *Mar Pollut Bull* 58:695–701.
12. de Castro ÍB, Perina FC, Fillmann G. 2012. Organotin contamination in South American coastal areas. *Environ Monit Assess* 184:1781–1799.
13. Gibbs P, Pascoe P, Burt G. 1988. Sex change in the female dog-whelk, *Nucella lapillus*, induced by tributyltin from antifouling paints. *J Mar Biol Assoc UK* 68:715–731.
14. Gibbs P, Bryan G. 1996. Reproductive failure in the gastropod *Nucella lapillus* associated with imposex caused by tributyltin pollution: A review. In Champ PFS, ed, *Organotin: Environmental Fate and Effects*, Vol 20. Chapman & Hall, Cambridge, UK, pp 259–278.
15. Gibbs P. 1996. Oviduct malformation as a sterilising effect of tributyltin (TBT)-induced imposex in *Ocenebra erinacea* (Gastropoda: Muricidae). *J Mollusc Stud* 62:403–413.
16. Horiguchi T, Kojima M, Kaya M, Matsuo T, Shiraishi H, Morita M, Adachi Y. 2002. Tributyltin and triphenyltin induce spermatogenesis in ovary of female abalone, Haliotis gigantea. *Mar Environ Res* 54: 679–684.
17. Horiguchi T, Kojima M, Takiguchi N, Kaya M, Shiraishi H, Morita M. 2005. Continuing observation of disturbed reproductive cycle and ovarian spermatogenesis in the giant abalone, *Haliotis madaka* from an organotin-contaminated site of Japan. *Mar Pollut Bull* 51:817–822.
18. Oehlmann J, Fioroni P, Stroben E, Markert B. 1996. Tributyltin (TBT) effects on *Ocinebrina aciculata* (Gastropoda: Muricidae): Imposex development, sterilization, sex change and population decline. *Sci Total Environ* 188:205–223.
19. Horiguchi T, Takiguchi N, Cho HS, Kojima M, Kaya M, Shiraishi H, Morita M, Hirose H, Shimizu M. 2000. Ovo-testis and disturbed reproductive cycle in the giant abalone, *Haliotis madaka*: Possible linkage with organotin contamination in a site of population decline. *Mar Environ Res* 50:223–229.
20. Horiguchi T, Kojima M, Hamada F, Kajikawa A, Shiraishi H, Morita M, Shimizu M. 2006. Impact of tributyltin and triphenyltin on ivory shell (*Babylonia japonica*) populations. *Environ Health Perspect* 114:13.
21. Cowie R. 2002. Apple snails (Ampullariidae) as agricultural pests: Their biology, impacts and management. In Baker G, ed, *Molluscs as Crop Pests*. CABI, Wallingford, UK, pp 145–192.
22. Rawlings T, Hayes K, Cowie R, Collins T. 2007. The identity, distribution, and impacts of non-native apple snails in the continental United States. *BMC Evol Biol* 7:97.
23. Hayes K, Joshi R, Thiengo S, Cowie R. 2008. Out of South America: Multiple origins of non-native apple snails in Asia. *Divers Distrib* 14:701–712.
24. Oscoz J, Tomás P, Durán C. 2010. Review and new records of non-indigenous freshwater invertebrates in the Ebro River basin (Northeast Spain). *Aquat Invasions* 5:263–284.
25. Giraud-Billoud M, Abud MA, Cueto JA, Vega IA, Castro-Vazquez A. 2011. Uric acid deposits and estivation in the invasive apple-snail, *Pomacea canaliculata*. *Comp Biochem Physiol A* 158:506–512.
26. Giraud-Billoud M, Vega IA, Rinaldi Tosi ME, Abud MA, Calderón ML, Castro-Vazquez A. 2013. Antioxidant and molecular chaperone defenses during estivation and arousal in the South American apple-snail *Pomacea canaliculata*. *J Exp Biol* 216:614–622.
27. Vega IA, Arribére MA, Almonacid AV, Ribeiro Guevara S, Castro-Vazquez A. 2012. Apple snails and their endosymbionts bioconcentrate heavy metals and uranium from contaminated drinking water. *Environ Sci Pollut Res Int* 19:3307–3316.
28. Gamarra-Luques C, Giraud-Billoud M, Castro-Vazquez A. 2013. Reproductive organogenesis in the apple snail *Pomacea canaliculata* (Lamarck, 1822), with reference to the effects of xenobiotics. *J Mollusc Stud* 79:147–162.
29. Giraud-Billoud M, Vega I, Carrion A, Castro-Vazquez A. 2010. Rudimentary male copulatory organs in females of an ampullariid snail. *Biocell* 34:49.
30. Shi H, Huang C, Zhu S, Yu X, Xie W. 2005. Generalized system of imposex and reproductive failure in female gastropods of coastal waters of mainland China. *Mar Ecol Prog Ser* 304:179–189.
31. Gamarra-Luques C, Winik BC, Vega IA, Albrecht EA, Catalan NM, Castro-Vazquez A. 2006. An integrative view to structure, function, ontogeny and phylogenetical significance of the male genital system in *Pomacea canaliculata* (Caenogastropoda, Ampullariidae). *Biocell* 30:345–357.
32. Estebenet AL, Cazzaniga NJ. 1998. Sex-related differential growth in *Pomacea canaliculata* (Gastropoda: Ampullariidae). *J Mollusc Stud* 64:119–123.
33. Estebenet A, Martín P. 2002. *Pomacea canaliculata* (Gastropoda: Ampullariidae): Life-history traits and their plasticity. *Biocell* 26:83–89.
34. Cazzaniga N. 1990. Sexual dimorphism in *Pomacea canaliculata* (Gastropoda: Ampullariidae). *Veliger* 33:384–388.
35. Albrecht E, Carreño N, Castro-Vazquez A. 1996. A quantitative study of copulation and spawning in the South American applesnail, *Pomacea canaliculata* (Prosobranchia: Ampullariidae). *Veliger* 39:142–147.
36. Koch E, Winik B, Castro-Vazquez A. 2009. Development beyond the gastrula stage and digestive organogenesis in the apple-snail *Pomacea canaliculata* (Architaenioglossa, Ampullariidae). *Biocell* 33:49–65.
37. Bauer B, Fioroni P, Ide I, Liebe S, Oehlmann J, Stroben E, Watermann B. 1995. TBT effects on the female genital system of *Littorina littorea*: A possible indicator of tributyltin pollution. *Hydrobiologia* 309: 15–27.
38. Matthiessen P, Gibbs PE. 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. *Environ Toxicol Chem* 17:37–43.
39. Burec-Drewniak W, Jaron I, Kucharzyk J, Narkiewicz W, Pasieczna A. 2013. Investigations of tin and molybdenum concentrations in the soils in the southern part of the Silesian Upland. [Cited 2013 July 25]. Available from: http://dx.doi.org/10.1051/e3sconf/20130108005.
40. Blunden S, Wallace T. 2003. Tin in canned food: A review and understanding of occurrence and effect. *Food Chem Toxicol* 41:1651–1662.
41. Duan L, Song J, Li X, yuan H, Xu S. 2012. Dissolved inorganic tin sources and its coupling with eco-environments in Bohai Bay. *Environ Monit Assess* 184:1335–1349.
42. Wang X, Fang C, Hong H, Wang W-X. 2010. Gender differences in TBT accumulation and transformation in *Thais clavigera* after aqueous and dietary exposure. *Aquat Toxicol* 99:413–422.
43. Bryan G, Gibbs P, Hummerstone L, Burt G. 1989. Uptake and transformation of ^{14}C-labelled tributyltin chloride by the dog-welk, *Nucella lapillus*: Importance of absorption from the diet. *Mar Environ Res* 28:241–245.
44. Oberdörster E, McClellan-Green P. 2002. Mechanisms of imposex induction in the mud snail, *Ilyanassa obsoleta*: TBT as a neurotoxin and aromatase inhibitor. *Mar Environ Res* 54:715–718.
45. Duft M, Schulte-Oehlmann U, Tillmann M, Weltje L, Oehlmann J. 2005. Biological impact of organotin compounds on mollusks in marine and freshwater ecosystems. *Coast Mar Sci* 29:95–110.
46. Janer G, Lyssimachou A, Bachmann J, Oehlmann J, Schulte-Oehlmann U, Porte C. 2006. Sexual dimorphism in esterified steroid levels in the gastropod *Marisa cornuarietis*: The effect of xenoandrogenic compounds. *Steroids* 71:435–444.
47. Grisolia C, Bicalho-Valadares M. 1997. Toxicity and genotoxicity of the fungicide triphenyltin hydroxide. *Rev Bras Genet* 20:243–246.
48. Estebenet A, Cazzaniga N. 1993. Egg variability and the reproductive strategy of *Pomacea canaliculata* (Gastropoda: Ampullariidae). *Apex* 8:129–138.
49. Giraud-Billoud M, Gamarra-Luques C, Castro-Vazquez A. 2013. Functional anatomy of male copulatory organs of *Pomacea canaliculata* (Caenogastropoda, Ampullariidae). *Zoomorphology* 132:129–143.
50. Takeda N. 2000. Development of a penis from the vestigial penis in the female apple snail, *Pomacea canaliculata*. *Biol Bull* 199:316–320.