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Morphological alterations in the freshwater rotifer *Brachionus calyciflorus* Pallas 1766 (Rotifera: Monogononta) caused by vinclozolin chronic exposure

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Abstract Vinclozolin (VZ) is a dicarboximide fungicide widely used on fruits, vegetables and wines, effective against fungi plagues. In this study we characterized the effects of VZ using a 4-day reproductive chronic assay with the freshwater rotifer *Brachionus calyciflorus*. The assay included observations of several features of asexual and sexual reproduction. Our results indicate that VZ: (a) increased asexual and sexual reproduction, (b) caused severe abnormality in females and (c) these abnormalities were inherited by sexual and asexual reproduction. At 1.2 mg/L three abnormal females were found out of 457 total females (0.66 %). This low percentage is consistent and reproducible according to further analysis, where we increased the number of replicates and total females exposed to 1.2 mg/L of VZ, and found 18 abnormal females out of 2868 total females (0.63 % abnormality). Interestingly, abnormal females found at 5.6 mg/L VZ exposure, were able to show mating behavior. Our results suggest that VZ behaves as a strong endocrine disruptor whose effects show the characteristic inverted-U-shape exposure concentration response curve regarding the intrinsic population increase and the percentage of abnormalities as endpoints.

Keywords Endocrine disruption · Abnormal rotifers · Transgenerational effects · Hormesis · Fungicide · Freshwater invertebrates

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Introduction

Environmental pollution by pesticides causes effects on human health and affects organisms at different biological levels. These effects are of critical global importance since Kohler and Triebskorn (2013) identified 120 pesticides as endocrine disruptors (ED), particularly to aquatic biota, where effects reported included: malformations, decline in growth, and mortality of embryos. Vinclozolin (VZ) is one of these pesticides that have been detected contaminating drinking water. Therefore, the Environmental Protection Agency (U.S. EPA) has conducted assessment of VZ principal metabolites M1 and M2, which are assumed to degrade completely to 3,5-dichlorophenyl in water (U.S. EPA 2000). The pesticide VZ [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-oxazolidine 2,4-dione] causes critical damage in species of invertebrates and vertebrates; it is a fungicide widely used on fruits, vegetables and wines (Spencer 1964; Papadopoulou-Marukidou 1991). It is one of several dicarboximide fungicides currently registered for use in the United States of America and in Europe (U.S. EPA 2003). It can be transported to surface water via spray drift from aerial and ground applications and may be available for runoff for several weeks to months after application (U.S. EPA 2000). VZ and its primary metabolites M1 and M2 are antagonists for androgen receptor, and are agonists for both estrogen receptors (ERα and ERβ) with a lower affinity for ERβ (Molina-Molina et al. 2006). Interestingly, these two metabolites display half-lives longer than 180 days and are likely highly mobile in the water phase, whereas VZ itself is not persistent (U.S. EPA 2000).

Most of the adverse effects of VZ have been studied in vertebrates: mainly mice, rats and fishes. The main effects in mice and rats include: (a) inhibit testosterone induced growth of androgen-dependent-tissues in mice and rats (Kang et al. 2004), (b) reduced spermatogenesis, testicular development, and prostate and liver of mice and rats (Uzumcu et al. 2004; Elzeinova et al. 2008; Guerrero-Bosagna et al. 2012). Sometimes these effects are inherited by their progeny, a phenomenon known as transgenerational inheritance (Anway et al. 2006).

In fishes there are fewer reports on the effects of VZ. In the rare minnow *Gobiocypris rarus* VZ caused: (a) decline of the renal somatic index, (b) presence of histopathological alterations on glomerulus and expansion of the Bowmańs space in kidneys, (c) elevated plasma cortisol concentration in females exposed to $\geq$2 µg/L VZ and males exposed to $\geq$10 µg/L VZ (Yang et al. 2011). In Japanese Medaka (*Oryzias latipes*) VZ has the potential to: (a) alter testicular development and gametogenesis (Kiparissis et al. 2003), and (b) produce significant decreases in male papillary processes in juveniles (Nakamura et al. 2014). In the guppy *Poecilia reticulata* VZ caused: (a) reduction on the orange display coloration, (b) inhibited gonopodium development, (c) reduced sperm count, and (d) suppressed courtship behavior (Bayley et al. 2002), in a manner consistent with anti-androgen action (Martinovic-Weigelt et al. 2011).

Endocrine disruption by VZ has been poorly studied in invertebrates (Lemos et al. 2010a). The terrestrial isopod *Porcellio scaber* was exposed to VZ in a 56-day reproductive assay, and chronic exposure effects were: (a) reduced pregnancy duration, (b) increased abortion percentage, (c) reduced number of pregnancies, and (d) reduced number of juveniles per female (Lemos et al. 2010b). Later, Lemos et al. (2010a) found delayed molting by VZ exposure in *P. scaber*. In *Daphnia magna* VZ induces a decrease in the number of newborn males at 1 mg/L, and the immobilizing effect (EC50, 48 h) of VZ for *D. magna* is 3.65 mg/L (Haeba et al. 2008). In mollusks VZ was shown to cause female virilisation (imposex development) and reduction of accessory sex organ expression in the freshwater snail *Marisa cornuarietis* and two marine prosobranchs *Nassarius lapillus* and *N. reticulatus* (Tillmann et al. 2001). Snails were exposed to nominal concentrations of VZ ranging from 0.03 to 1 µg/L for up to 5 months. In low concentration exposed juvenile *M. cornuarietis* males had a slight decrease in the penis and penis sheath that was reversible once they attained puberty. Adult male *N. lapillus* exposed to the fungicide developed shorter penis, smaller prostate gland, and there were less males with ripe sperm stored in the seminal vesicle. Compared to the reported effects of estrogens and androgens on these two same species, these anti-androgenic responses seem to be less drastic, and might not have any biological effect at population level (Tillmann et al. 2001). The immobilizing effect (EC50, 48 h) of VZ on American oyster (*Crassostrea virginica*) was reported to be 3.2 mg/L (Haeba et al. 2008). For the opossum shrimp (*American bahia*) the LC50 (96 h) was 1.5–2.1 mg/L VZ (U.S. EPA 2000).

In the crustacean copepod *Acartia tonsa* the response of the male gonad to $\geq$0.10 mg/L VZ caused alterations in spermatogenesis. Spermatophore formation was affected leading to deformations. In VZ exposed *A. tonsa* females no statistically significant effects were observed (Watermann et al. 2011).

To our knowledge, no data on morphological alteration in rotifers due to toxicant chronic VZ exposure has been reported in the mainstream scientific literature. We found a report of a LC50 = 30.5 mg/L for VZ in the rotifer *Brachionus calyciflorus*; and chronic effects in the range of VZ 0.185–3.0 mg/L exposure, with results showing an inverse U shape response characteristic of endocrine disruption (ED) (Zavala-Aguirre et al. 2007). Rotifer reproduction has been suggested as a sensitive endpoint for ED assessment (Preston et al. 2000). Rotifers are one of the most important components of freshwater biota and play an important role in aquatic ecosystems. Their ubiquity and abundance explain their standing as one of the three main groups of freshwater aquatic ecosystems (Wallace et al. 2006). *Brachionus calyciflorus* belongs to a species complex in which some geographically and genetically distinct strains are reproductively isolated from one another (Gilbert and Walsh 2005). Rotifers are good model organisms for studies of the effects of pesticides on aquatic ecosystems, where they occupy an important place in many trophic webs. The freshwater rotifer *B. calyciflorus* shows a heterogonic life cycle in natural behavior, in the asexual reproduction the amictic females produce eggs that develop by diploid parthenogenesis. Sexual reproduction is initiated when mictic females produce offspring which are not differentiated morphologically of the amicitc females; mictic females produce resting eggs or cysts when their haploid eggs are fertilized. The unfertilized haploid eggs produce males (Dahms et al. 2011; Gilbert 2004). VZ damages reproductive organs of vertebrates and invertebrates. These effects can be easily observed, quantified, and studied in rotifers. Therefore, we aim to assess ED effects caused by chronic VZ exposure in the freshwater rotifer *B. calyciflorus*, using a reproductive assay that considers asexual and sexual reproduction, and to investigate morphological abnormalities and their frequency in all phases of its life cycle, and if such abnormalities could be inherited to subsequent generations of laboratory cultured rotifers.

Materials and methods

Rotifer collection and culture

The freshwater rotifer *Brachionus calyciflorus* (Pallas 1766) originally collected in Gainsville, Florida, USA, and has been cultured continuously in the laboratory of Dr. Terry W. Snell for over 20 years; were cultured in a bioclimatic chamber with a 16:8 dark: light period and a temperature of 25 ± 2 °C (Perez-Legaspi and Rico-Martínez 1998). Rotifers were kept in Petri dishes with EPA medium (U.S. EPA 1985), and fed the green alga *Nannochloris oculata* (strain LB2194 of the University of Texas Collection) grown in Bolds Basal Medium (Nichols 1973). Typically 10–15 Petri dishes containing each from 100 to 500 rotifer females were kept.

Reproduction test

VZ (Pestanal®: CAS No. 5047-44-8: $C_{12}H_9Cl_2NO_3$) was screened for effects on asexual and sexual reproduction in *B. calyciflorus* using a 4-day reproduction test. We placed 5 neonates (<24 h old) in 2 mL EPA medium (negative control) with 5×10^6 cells/mL of the green alga *Nannochloris oculata* (strain LB2164 from University of Texas Collection); this protocol represents a slight modification from that of Snell and Moffatt (1992), because we used 24-well polystyrene plates (Costar Co., USA) instead of test tubes. Test conditions are the same in both protocols: 25 °C, pH 7.4–7.8, hardness 80–100 mg $CaCO_3$/L. Six replicates were performed per control, solvent control (1 % acetone from Supelco Co: Bellefonte, PA, USA, which was the highest concentration used to dissolve VZ), and the VZ concentrations: 0.4, 0.8, 1.2, 1.6, 2.0, 2.8, 5.6, 12.8 and 18.0 mg/L (nominal concentrations). For the 1.2 mg/L VZ concentration we made another 21 replicates. All VZ concentrations are lower that the LC50 = 30 mg/L reported by Zavala-Aguirre et al. (2007). At the end of the test, total rotifers and eggs were placed into a glass Petri dish and the number of nonovigerous females, ovigerous asexual females, ovigerous unfertilized sexual females, ovigerous fertilized sexual females, males, parthenogenetic (asexual or amictic) eggs, unfertilized sexual eggs, fertilized sexual eggs (cysts) were counted. The intrinsic growth rate (r) of females, were expressed according to the formula: $r = (\ln N_t - N_o)/T$, where $\ln N_t$ = natural logarithm of the number of female in the plate after 4 days, $\ln N_o$ = natural logarithm of the initial number of rotifers in each plate (only used the N_o for females because the initial organism were five). For nonovigerous females, ovigerous asexual females, ovigerous unfertilized sexual females, ovigerous fertilized sexual females, male, parthenogenetic (asexual or amictic) eggs, unfertilized sexual eggs, fertilized sexual eggs (resting eggs) were expressed a total found at the end of the experiments. Ovigerous females were classified by the morphology of their eggs, which differ between females, male and resting eggs (Wallace et al. 2006). The abnormal rotifers were observed at 20x magnification in a Leica DMLS inverted microscope. Photographic and video recordings were made to study abnormalities and to measure length and width in rotifers only at the 1.2 mg/L VZ concentration (which is the VZ concentration with the highest percentage of abnormal females found). We selected 20 males and 10 females (from the first six replicates) that presented a big vitellarium with an Infinity 3 camera and Infinity Capture 4.6.0 software (Lumenera Co., Ottawa, ON, Canada). Measurements from these females and males were compared only with those of controls. We performed cross-mating assays according to the protocol of Rico-Martinez and Snell (1997), only with the abnormal females and males found in the concentration of exposure to 1.2 mg/L VZ to verify and quantify: mating attempts, copulations, and cyst production (only 1 replicate: see annex: video recordings). Also, we obtained cysts (n = 155) after exposure to 1.2 mg/L VZ according to previous results (cysts collected from the extra 21 replicates), and we recorded the hatching percentage of cysts produced by abnormal females and compared it with that of cysts produced by normal animals (control). The cysts collected from the 1.2 mg/L VZ exposure experiment were washed six times with EPA medium to eliminate any traces of VZ from the cyst surface. Then the cysts were incubated for 10 days at 4 °C in darkness to inhibit hatching. After incubation period, 10 cysts were placed in 1 mL EPA medium in individual wells of 24-well polystyrene plates. The plates were placed in bioclimatic chamber at 25 ± 2 °C and 16:8 light:darkness photoperiod to induce hatching. Finally after 24 h we counted the number of hatched cysts and compare them with the number of cysts produced in normal culture conditions. To the rotifers hatched from this experiment (control and those produced under VZ exposure), we evaluated the intrinsic growth rate (*r*). Additionally, we carried out the same experiment including more replicates for 1.2 mg/L

VZ to demonstrate the reproducibility of the low abnormality percentage (n = 21).

Scanning electron microscopy

We collected 18 abnormal rotifers and placed them in an Eppendorf tube in a final volume of 1 mL of EPA medium and added 100 µL of 3 % formalin. The samples were dehydrated with alcohol (60–100 %), moisture was removed by a critical point dryer (Tousimis) with liquid CO_2. Samples were then coated with gold using a DESK II chamber, and photographed with JEOL 5000 LV SEM.

Statistical analysis and interpretation of data

Statistical analyses were performed with Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA). We used one way ANOVA and post hoc Duncańs comparison tests. The use of solvents like acetone had no adverse effects on *B. calyciflorus* reproduction.

Results

VZ increased 17.90 % on average the intrinsic growth rate (*r*) in amictic females in all chronic exposure concentrations tested except at 5.6 mg/L respect to the control and solvent control (solvent control was 0.89 ± 0.03) (Fig. 1). In addition, VZ increased the total net production of males, females with parthenogenetic eggs, females with sexual unfertilized eggs, parthenogenetic eggs, and sexual unfertilized eggs. VZ cause abnormal females in an exposure interval of 0.4–5.6 mg/L (Table 1). At 1.2 mg/L VZ we found the highest production of abnormal females, a total of 3 organisms that represent 0.66 % of total females counted (457) in 6 replicates. In another 21 replicates (in the same exposure regimen of 1.2 mg/L VZ) we found 18 abnormal females out of 2868 total females counted which represent 0.63 % of VZ exposed female population. Table 1 shows the percentage of abnormal rotifers found in all experiments, when the rotifers were intoxicated with VZ. We also observed different types of abnormalities in all these rotifers with scanning electronic microscopy and observed marked deformities as: (a) enormous vitellarium (Fig. 2b), (b) dwarfism (Fig. 2c, c1), (c) twisted spines (Fig. 2d, d1), (d) squared appearance of rotifer body (Fig. 2d), (e) mastax apparently deformed (Fig. 2e), (f) deformed cuticle (Fig. 2e1), (g) feet attached to head (Fig. 3b, b1), (h) vestigial foot or without foot (Fig. 3c, c1), (i) three toes in the foot (normally two toes are found) (Fig. 3d1), (j) curved body (Fig. 3d, d2) and (k) deformed spines (Fig. 3e, e1). At 1.2 mg/L. VZ we observed the highest reproductive effect; while at 0.8 and 1.2 mg/L VZ we noted increments of total production of males compared with control and other treatments (Table 2). We also noted differences in maximum length and width (p = 0.04 and 0.01 respectively) in 20 males intoxicated at 1.2 mg/L VZ when compared with 20 males produced in the negative control (Table 3), although no deformities were found in males. The ten females (produced in the same exposure regimen of 1.2 mg/L VZ, first 6 replicates) with the abnormal vitellarium were more robust than those of the negative control, especially in the region around the vitellarium (see Fig. 2b and measurements in the Table 3). We found significant differences in the total production of amictic ovigerous females only at 2.8, 12.8 and 18.0 mg/L VZ exposure, and at 2.0 mg/L for ovigerous fertilized sexual females (Table 2). The number of eggs only showed significant differences for unfertilized sexual eggs at 2.8 mg/L and parthenogenetic eggs at 18 mg/L (Table 2). Fascinatingly, the abnormal females exposed to VZ 5.6 mg/L are capable of performing complete sequences of mating behavior (see video recordings of the annex). Additionally we recorded the movement of one representative abnormal female produced in the VZ chronic exposure experiments, for almost all concentrations used. These females had a characteristic behavior: they only moved in circles and apparently lacked synchrony of their bodies, they lived only 2 days, and were unable to feed or to assimilate food (J. A.-F. pers. observations).

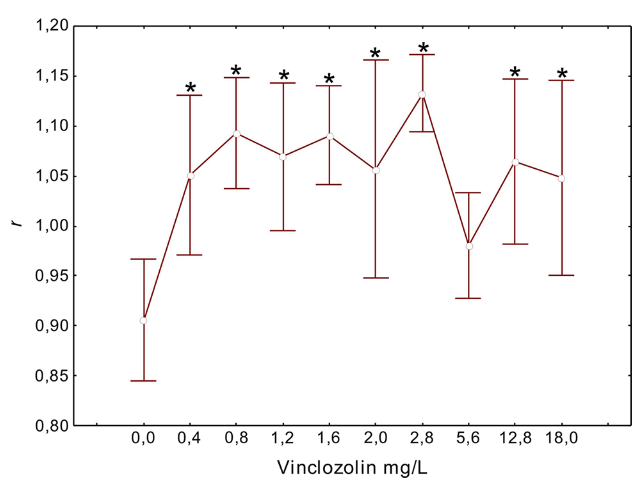

Fig. 1 Effect of vinclozolin on population growth rate (r). Vertical lines on columns indicate ± one standard deviation, *asterisk* indicate significant differences (p = 0.05) from control and solvent control (r = 0.89 ± 0.03)

To demonstrate the epigenetic inheritance of the abnormalities, we tested and evaluated the hatching percentage of cysts produced with 1.2 mg/L vinclozolin. Surprisingly, the hatching percentage was 64.52 % (n = 155), which compared well to the hatching percentage of cysts obtained under normal culture conditions

Table 1 Abnormality percentages and occurrence in the VZ exposure chronic assay using the freshwater rotifer *B. calyciflorus*

Vinclozolin (mg/L)	Total females (#)	Abnormal females (#)	Abnormal females (%)	Replicates
0 (control EPA)	261	0	0	6
0 (solvent control)	250	0	0	6
0.4	540	2	0.37	6
0.8	608	1	0.16	6
1.2	457	3	0.66	6
1.2*	2868	18	0.63	21
1.6	474	1	0.21	6
2.0	477	1	0.21	6
2.8	664	1	0.15	6
5.6	511	1	0.2	6
12.8	475	0	0	6
18.0	453	0	0	6

The asterisk indicates more replicates in this concentration

(EPA control) of 60 % (n = 120). Both rotifers after hatching from cysts were evaluated on their intrinsic growth rate (*r*) without VZ exposure during the 4 days test. The *r* of the rotifers hatching from cysts produced by VZ exposure (1.2 mg/L) was 1.21 ± 0.063 (mean ± one SD), and the *r* of the rotifers hatching from control cysts was 0.90 ± 0.064 (solvent control was 0.89 ± 0.03). Therefore, VZ improved the intrinsic growth rate in the rotifers hatching from cysts after VZ exposure, compared with rotifers hatching from cysts. The rotifers hatching from control and solvent control cysts did not produced abnormal rotifers. The rotifers hatching from cysts produced by VZ exposure (1.2 mg/L) and after 4 days culture without VZ produced 4 abnormal females out of 1670 total rotifers (12 replicates). These abnormal females represent the 0.24 % of total females. These abnormal females were produced from cysts that were: (a) carefully removed and washed after VZ exposure, (b) incubated in darkness at 4 °C, (c) then placed the cysts in groups of ten, and (d) then monitored the hatching of the cysts produced during 1.2 mg/L VZ exposure, and after 24 h of incubation, and observing the females for their entire lifespan, recorded absence of abnormal females in the F1 generation (after 24-h all cysts hatched). Therefore, these results suggest that these abnormalities are inherited to the next generations of rotifers.

Discussion

The toxicity of VZ to *B. calyciflorus* is consistent with the action of a strong ED, since it increases asexual and sexual reproduction, and cause severe deformities in the body of rotifers at chronic exposure concentrations. VZ significantly increased female production in the exposure range of 0.40–18 mg/L. In both cases (effect on the *r* value and percentage of abnormal females), the maximum effects are found at intermediate concentrations showing an inverted-U-shape exposure concentration/response curve (also known as non-monotonic curve) (Welshons et al. 1999). In fact Zavala-Aguirre et al. (2007) exposed the rotifer *B. calyciflorus* for 96-h with 0.185–3.0 mg/L VZ in a sublethal test and their results showed an inverse U shape. These “non-monotonic” or U- or inverted U-shaped dose–response curves associated with “low dose” effects have been explained based on endocrinological principles (Welshons et al. 1999). This effect is known as hormesis and is the name given to the stimulatory effects caused by low levels of potentially toxic agents (Stebbing 1982). In this context any reproductive toxicant capable of endocrine disruption can be considered an “endocrine-disrupting chemical” (“EDC”) or an “ED” (Evans 2011). The shape of the dose–response curves for these effects varies with the endpoint and dosing regimen, and may be low-dose linear, threshold-appearing, or non-monotonic (USEPA 2001).

Regarding the increases in asexual and sexual reproduction, we observed a synchrony in the increase of females and males due to the effect of VZ exposure. This synchrony of males and sexual females (which are capable of being copulated and fertilized), is known as the haplodiploid system (Gómez 1996); this means that in the initial stages of the sexual phase, the density of males is so low that there are practically no fertilized females, and the progeny of the unfertilized females produce an increase in males. As soon as the male density increases this feedback system would result in an increase of fertilized females, then decreasing male production and increasing the number of cysts (fertilized eggs) (Gómez 1996).

To our knowledge, this is the first report in rotifers, where there is demonstration of abnormal morphologies in female and increase in length and width of males caused by chronic VZ exposure. Our results of abnormalities found in females of the rotifer *B. calyciflorus* include: (a) females

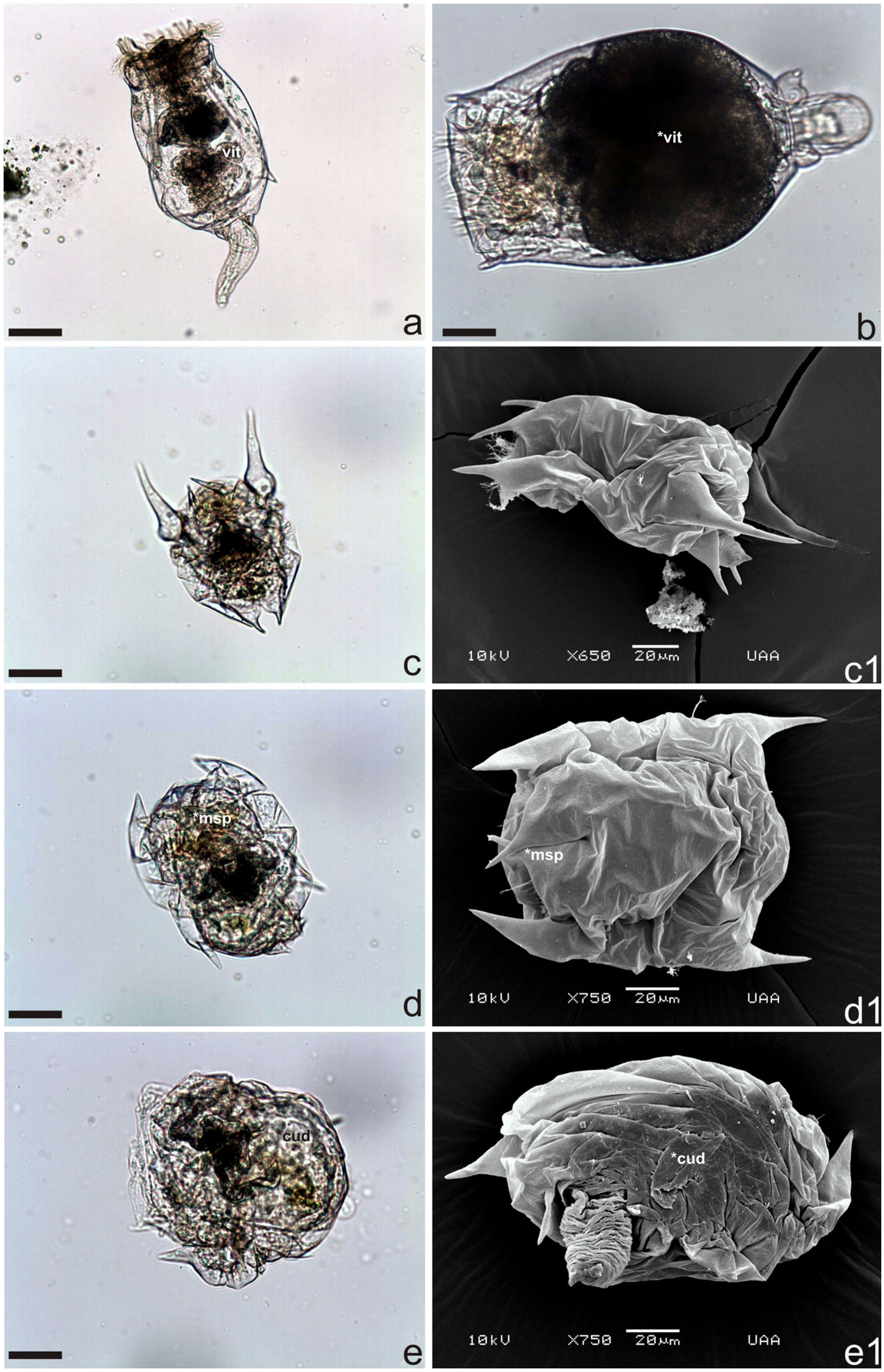

Fig. 2 Morphological alterations in females in the rotifer *Brachionus calyciflorus* by vinclozolin 1.2 mg/L. **a** Normal female with usual vitellarium; **b** female with enormous vitellarium (*vit), **c** and **c1** dwarfism female, **d** and **d1** median spines (*msp) curves in females; **e** and **e1** cuticule deform (*cud) in females. The *black bar* at the posterior margin of each figure equals 100 μm

with an enormous vitellarium, (b) twisted spines, (c) feet attached to the head in the part of the trochal disk, that caused a curved body, (d) mastax probably deformed with strange movements (see video recordings in the annex), squared appearance of female body, (e) dwarfism in females, (f) presence of granules that only are present in male

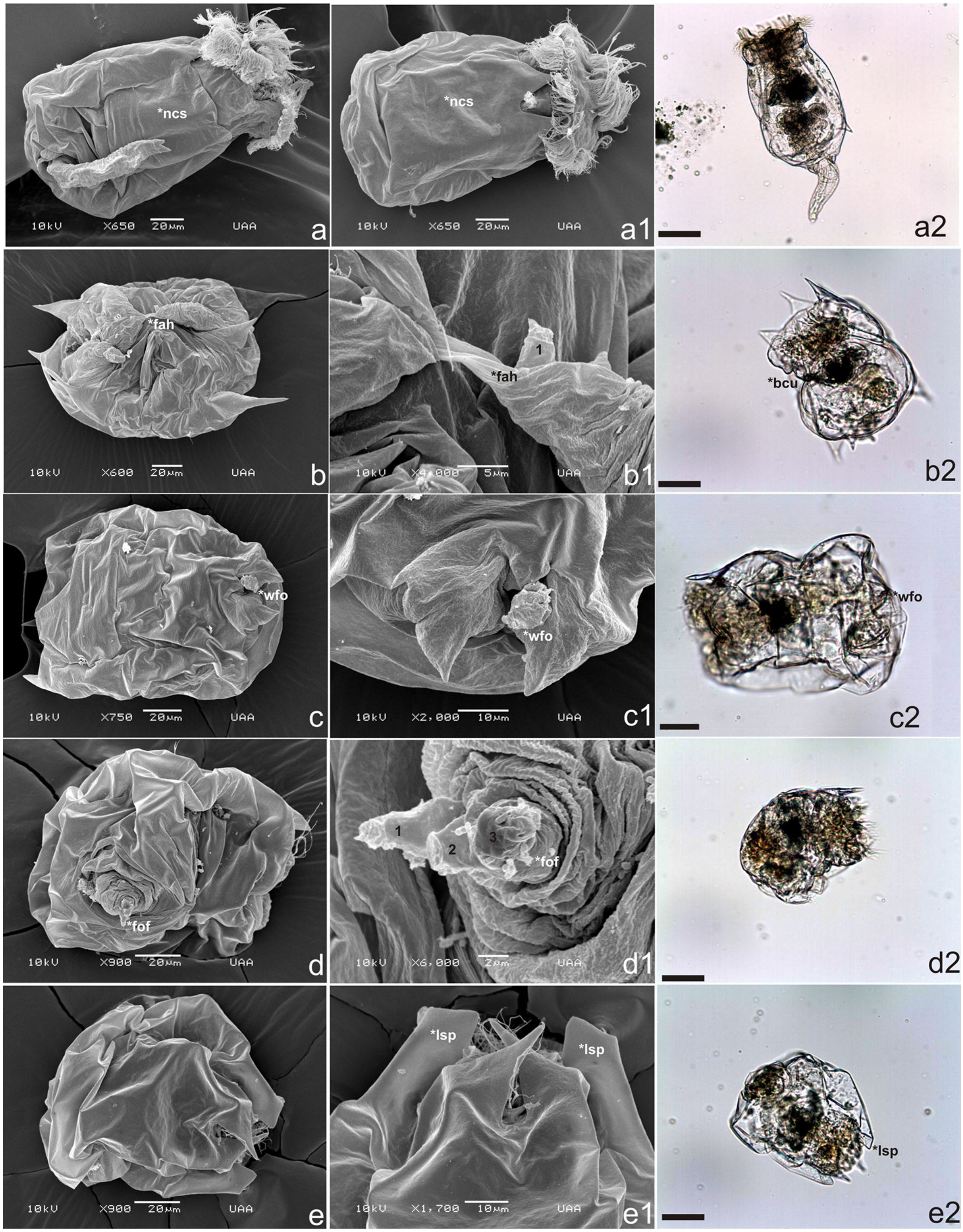

Fig. 3 Morphological alterations in females in the rotifer *B. calyciflorus* by vinclozolin 1.2 mg/L. **a**, **a1** and **a2** Normal female showeing normal cuticle surface (*ncs); **b**, **b1** and **b2** rotifer body curved (*bcu) and foot attached to the head (*fah); **c**, **c1** and **c2** females without foot (*wfo); **d**, **d1** and **d2** foot with three fingers (*fof); and **e**, **e1** and **e2** lateral spines (*lsp) deforms and splices in females. The *black bar* at the posterior margin of each figure equals 100 µm. The *number in black* indicates the number of feet of the foot

(a possible masculinization) and, (g) increase of maximum length and width in males exposed to 1.2 mg/L. VZ is known to bind androgen receptor; in fact, is an antagonist to progesterone receptor (Molina-Molina et al. 2006), and recently, Snell and DesRosiers (2008) demonstrated in rotifers the presence of a membrane associated progesterone receptor that suggest that progesterone-like ligand may play a role in regulating reproduction (Snell 2011). In

Table 2 Effects of VZ on sexual and asexual reproduction in the rotifer *B. calyciflorus*: total males, total females with parthenogenetics eggs (fpe); total females with sexual eggs unfertilized (fseuf); total females with sexual eggs fertilized (fsef); total parthenogenetics eggs (pe); total sexual eggs unfertilized (seuf); total sexual eggs fertilized or cysts (sef)

Vinclozolin (mg/L)	Males	fpe	fseuf	fsef	pe	seuf	sef
0 (control EPA)	2 ± 2.6	14.66 ± 5.78	2.16 ± 1.16	0	20.66 ± 8.23	6.66 ± 47.6	0
0 (solvent control)	12 ± 13.3	18.33 ± 3.98	1.17 ± 0.75	0	21.33 ± 5.01	6.83 ± 6.62	0
0.4	16.83 ± 16.79	16.5 ± 5.95	3 ± 0.89	1.33 ± 1.86	20 ± 6.16	12.83 ± 11.63	1.33 ± 1.86
0.8	36.83 ± 45.50*	15.16 ± 3.18	3.16 ± 2.48	1.5 ± 2.5	17.33 ± 5.46	12 ± 16.87	1.5 ± 2.5
1.2	36.83 ± 25.34*	14.66 ± 4.5	1.83 ± 1.6	1.33 ± 1.75	18.5 ± 7.66	8.83 ± 8.9	1.33 ± 1.75
1.6	30.5 ± 18.47	12.16 ± 5.45	2.83 ± 2.63	2.66 ± 4.84	14.5 ± 8.64	9.33 ± 15.46	2.83 ± 5.23
2.0	35.33 ± 27.08	11.83 ± 5.11	3 ± 2.89	3.16 ± 2.48	14.66 ± 7.5	10.16 ± 13.56	3.33 ± 2.8
2.8	19 ± 17.75	28.66 ± 5.57*	9.16 ± 5.03*	0	33.16 ± 8.47	54.33 ± 32.98*	0
5.6	4.16 ± 4.4	21.16 ± 4.02	2.5 ± 1.76	0	28.16 ± 14.99	17.83 ± 16.44	0
12.8	14.16 ± 11.72	24.5 ± 10.25*	5.83 ± 1.72	0	31.83 ± 11.44	24.16 ± 11.26	0
18.0	6.66 ± 8.66	26.33 ± 8.01*	4 ± 4.33	0	38.16 ± 8.51*	17.83 ± 17.55	0

The values are the media ± one standard deviation, asterisk indicate significant differences ($p = 0.05$) from control and solvent control

Table 3 Morphometric analysis of normal females and males (control negative produced) against abnormal females (with enormous vitellarium) and males produced at 1.2 mg/L VZ exposure

	Maximum length (μm)	Maximun width (μm)
Normal females	543.68 ± 110.66	354.72 ± 97.83
Abnormal females 1.2 mg/L Vz	765.48 ± 27.26	476.47 ± 25.6
p value	0.0002	0.01
Normal males	317.67 ± 19.14	223.96 ± 15.95
Abnormal males 1.2 mg/L Vz	332.95 ± 24.88	236.72 ± 14.33
p value	0.04	0.01

The values are the media ± one standard deviation

fact, progesterone is a steroid that plays a key role in reproduction of variety of vertebrates and invertebrates (Graham and Clarke 1997), and the abnormalities found in rotifer females could be explained because of the toxicity mechanism of VZ on disturbing rotifer development, probably affecting: (a) ovaries, (b) vitellarium (yolk gland), (c) oviduct, and eggs of females, and seminal vesicle, (d) rudimentary gut, and (e) sperm duct of males, as progesterone receptors in rotifers are found in all these structures in rotifers (Stout et al. 2010). We suggest that these abnormalities affect directly: (a) mating behavior, (b) reproduction, (c) feeding, and (d) normal development in rotifers.

Interestingly, the abnormalities were found at the end of the reproductive assay, at low frequencies. At 1.2 mg/L we detected the maximum number of abnormal females (Table 2). VZ induce transgenerational alteration, which could be explained as a transgenerational inheritance of environment-induced epigenetic changes in non-exposed subsequent generations (Skinner et al. 2011). In these assay the rotifers are exposed all the time, but not all the rotifers presented abnormalities. In fact, at 1.2 mg/L VZ exposure we found 3 abnormal females out of 457, that represents the 0.66 % of total females observed, compared to the control and solvent control (acetone) where abnormal rotifers were not found. In the second experiment we found 18 abnormal females out of 2868 that represents 0.63 % from total females observed. In this context, VZ probably modifies the epigenome of the germ line permanently, and then the promoted disease could become transgenerationally transmitted to subsequent progeny (Skinner et al. 2011). We suggest this mode of action for VZ in rotifers, due to our results where cysts produced by females previously exposed to 1.2 mg/L VZ were carefully washed, and when the cysts hatched produced a F1 generation of rotifer females lacking abnormalities. However, abnormalities were evident after 4 days in the next parthenogenetic generations. However, the cysts are produced by induction of sexual reproduction. In this context, the effect of VZ can be inherited to next generations by both asexual and sexual routes.

What is the mechanism for producing such a low frequency of abnormal females? In rats exposed to VZ, Anway et al. (2005) mention that the abnormal phenotype transmission is associated to the male germ line. Perhaps the females showing abnormalities are those produced sexually from altered males after exposure to VZ in our experiment.

Since rotifers reproduce mainly by parthenogenesis, and both field and laboratory populations are composed mainly of females this could in part explain the low incidence of abnormal females that we recorded in our experiments. It is important to mention that even in vertebrates there is still controversy about the effects of VZ producing abnormalities or even lesser effects. Schneider et al. (2008) studied a possible transgenerational effect of the fungicide VZ on the male reproductive system following oral exposure since this effect was reported by Anway et al. (2005). The exposure was in pregnant wistar rat by oral gavage with vinclozolin 0, 4 or 100 mg/(kg bw day). They concluded that no transgenerational effect on male reproductive system was found. They also reported VZ exposure in utero had no statistically significant effect on reproduction in terms of sexual maturity (age preputial separation), time to mate, or mating and fertility indices, either utero-exposed F1 offspring or in subsequent generations (F2 and F3). This suggests that the mechanism of action of VZ is not understood yet, and the frequency of alterations in vertebrates might be too low.

The vitellarium in females exposed to 1.2 mg/L VZ increased more than the normal vitellarium in control females. This in turn, increased the size of females. Males also increased their size at 1.2 mg/L VZ exposure. Similar phenomena (although in different direction) have been reported in other aquatic invertebrates and vertebrates exposed to VZ. For example, in *Daphnia magna* exposed to 0.43 mg/L VZ induced a small decrease in body length (Vandegehuchte et al. 2010). Females of the fish medaka (*Oryzias latipes*) decrease the total length at 5 mg/L VZ, and males increased total length (Kiparissis et al. 2003). VZ affected the normal development of body, causing abnormalities at chronic exposure.

Abnormalities allegedly caused by toxicants have been reported in several species of cladocerans. Elmoor-Loureiro (2004) found abnormalities in the postabdomen of the cladoceran *Ilycryptus spinifer* in samples collected at the Apipucos Reservoir at Recife, Brazil. He argues that occurrence of this abnormality-inducing environmental factor is occasional or its concentration in water should be variable, as suggested by the absence of abnormalities in the youngest ones, and in the totality of the individuals from different samples. Otha et al. (1998) found abnormalities in the *Daphnia* carapace by exposure to ethylenethiourea, a carcinogenic metabolite of ethylene-bisdithiocarbamate fungicides. Shurin and Dodson (1997) tested the effects of nonylphenol, a non-ionic surfactant common in sewage effluents, on *Daphnia*. They showed that, under experimental conditions, nonylphenol at environmentally relevant concentrations could produce malformations in *Daphnia* postabdomen and antenna.

VZ treated females in all concentrations had more offspring, and total males produced per amictic and mictic female than those of the controls. Other toxicants with effects on endocrine systems like fenitrothion (competitive androgen receptor antagonist) in the rotifer *B. calyciflorus* caused disruption on reproduction: females had longer reproductive periods and produced more offspring than controls, also reduced the number of resting eggs produced, and low concentrations of fenitrothion increased the duration of the juvenile period, reproductive period, and lifetime reproduction (Lv et al. 2010). Zou (2003) review the effects of endocrine disruption in invertebrates describing a large list of toxicants with ED effects in rotifers. Pentachlorophenol, and chlorpyrifos reduced the sexual reproduction in the rotifer *Brachionus*; diazinon, fenitron, isoprotiolano, methoprene increase the production of resting eggs, and finally; nolylphenol and precocene inhibited the fertilization in females. However, in all of these experiments they did not report alterations in morphological characteristics of rotifers, as we did in this work.

The exposure concentrations tested are environmentally relevant based on the following considerations regarding environmental concentrations of VZ:

1. The U.S. EPA cited VZ has a degradation product with properties and characteristics associated to chemicals detected in ground water. The use of this chemical in areas where soils are permeable, particularly where the water table is shallow, may result in ground water contamination (U.S. EPA 2000).
2. The use of VZ in the winemaking industry is evident, and the levels of VZ in grapes before processing for red wine is 1.13 mg/kg and at the end of the process is 0.057 mg/kg VZ (Navarro et al. 1999). Red wine is probably a source of contamination of acute and chronic direct exposure to humans, and even more problematic is the fact that contaminated grapes are consumed directly without any relevant quality control.
3. VZ were detected in France in ground water at 0.06 μg/L (average 160.5 ppb) (Van Den Berg and Van Der Linden 1994; El Shahat et al. 2003) and surface water at 128–390 ppb (El Shahat et al. 2003); in Australia in surface water at 0.01–0.10 μg/L (Thoma and Nicholson 1989); in Germany in surface water at 2.4 μg/L (Oskam et al. 1993); in the Netherlands in rain water samples at a maximum concentration of 28 ng/L (Siebers et al. 1994); in Jordan in sediments/soil at concentrations ranging from 7.3 to 116.0 ppb (Al-Mughrabi and Qrunfleh 2002).

Conclusions

The effects produced by chronic exposure to VZ in rotifers are evident, and when we compare all of these effects with those reported in vertebrates and invertebrates, we found several similarities:

(a) VZ cause abnormalities in both invertebrates and vertebrates,
(b) VZ is able to inherit the adverse effects to subsequent generations in vertebrates and invertebrates, but is more evident on invertebrates because the life cycle on invertebrates is faster than vertebrates,
(c) VZ altered the normal size of vertebrates and invertebrates.

Our results are consistent with an EDC mode of action in rotifers exposed at environmentally relevant concentrations and caused some adverse effects which are comparable with those observed in vertebrates exposed to VZ.

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Conflict of interest The authors report no declarations of interest.

References

Al-Mughrabi KI, Qrunfleh IM (2002) Pesticides residues in soil from the Jordan Valley, Jordan. Bull Environ Contam Toxicol 68:86–96

Anway MD, Cupp AS, Uzumcu M, Skinner MK (2005) Epigenetic transgenerational actions of endocrine disruptions and male fertility. Science 308:1466–1469

Anway MD, Memon MA, Uzumcu M, Skinner MK (2006) Transgenerational effect of the endocrine disruptor vinclozolin on male spermatogenesis. J Androl 27:868–879

Bayley M, Junge M, Baatrup E (2002) Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. Aquat Toxicol 56:227–239

Dahms HU, Hagiwara A, Lee JS (2011) Ecotoxicology, ecophysiology, and mechanistic studies with rotifers. Aquat Toxicol 101:1–12

Den Berg Van, Der Linden Van (1994) Agricultural pesticides and groundwater. In: Zoller U (ed) Groundwater contamination and control. USA Marcel Dekker, New York, pp 293–313

Elmoor-Loureiro LMA (2004) Morphological abnormalities in the cladoceran *Ilyocryptus spinifer* (Apipucos Reservoir, Pernanmbuco State, Brazil). Braz J Bio 64:53–58

El-Shahat MF, Al-Nawayseh KM, Jiries AG, Alnasir FM (2003) Pesticides and heavy metal distribution in southern Dead Sea basin. Bull Environ Contam Toxicol 71:1230–1238

Elzeinova F, Novakova V, Buckiova D, Kubatova A, Peknicova J (2008) Effect of low dose of vinclozolin on reproductive tract development and sperm parameters in CD1 outbred mice. Reprod Toxicol 26:231–238

Evans TJ (2011) Endocrine disruptors. In: Gupta RC (ed) Reproductive and developmental toxicology. Academic Press, San Diego, pp 873–891

Gilbert JJ (2004) Females from resting eggs and parthenogenetic eggs in the rotifer *Brachionus calyciflorus*: lipid droplets, starvation resistance and reproduction. Freshw Biol 49:1505–1515

Gilbert JJ, Walsh EJ (2005) *Brachionus calyciflorus* is a species complex: mating behavior and genetic differentiation among four geographically isolated strains. Hydrobiologia 546:257–265

Gómez GA (1996) Ecología genética y sistemas de reconocimiento de pareja en poblaciones simpátricas de rotíferos. Tesis doctorales. ETD Micropublicaciones, Universitat de Valéncia

Graham JD, Clarke CL (1997) Physiological action of progesterone in target tissue. Endocr Rev 18:502–519

Guerrero-Bosagna C, Covert TR, Haque MdM, Settles M, Nilsson EE, Anway MD, Skinner MK (2012) Epigenetic transgenerational inheritance of vinclozolin induced mouse adult onset disease and associated sperm epigenome biomarkers. Reprod Toxicol 34:694–707

Haeba MH, Hilscherová K, Mazurová E, Bláha L (2008) Selected endocrine disrupting compounds (vinclozolin, flutamide, ketoconazole and dicofol): effects on survival, occurrence of males, growth, molting and reproduction of *Daphnia magna*. Environ Sci Pollut Res Int 15:222–227

Kang IH, Kim HS, Shin JH, Kim TS, Moon HJ, Kim IY, Choi KS, Kil KS, Park YI, Dong MS, Han SY (2004) Comparison of anti-androgenic activity of flutamide, vinclozolin, procymidone, linuron, and p, p′-DDE in rodent 10-day Hershberger assay. Toxicology 199:145–159

Kiparissis Y, Metcalfe TL, Balch GC, Metcalfe CD (2003) Effects of the antiandrogens, vinclozolin and cyproterone acetate on gonadal development in the Japanese medaka (*Oryzias latipes*). Aquat Toxicol 63:391–403

Köhler H-R, Triebskorn R (2013) Wildlife ecotoxicology of pesticide: can we track effects to the population level and beyond? Science 341:759–765

Lemos MFL, Van Gestel CAM, Soares AMVM (2010a) Reproductive toxicity of the endocrine disruptor vinclozolin and bisphenol A in the terrestrial isopod *Porcellio scaber* (Latreille, 1804). Chemosphere 78:907–913

Lemos MFL, Van Gestel CAM, Soares AMVM (2010b) Developmental toxicity of the endocrine disruptors bisphenol A and vinclozolin in a terrestrial isopod. Arch Environ Contam Toxicol 59:274–281

Lv LL, Snell TW, Yang JX, Li SG, Zhu WG (2010) Effect of fenitrothion on life history parameters of the rotifer *Brachionus calyciflorus*. J Freshw Ecol 25:589–598

Martinović-Weigelt D, Rong-Ling W, Villeneuve DL, Bencic DC, Lazorchak J, Ankley GT (2011) Gene expression profiling of the androgen receptor antagonists flutamide and vinclozolin in zebrafish (*Danio rerio*) gonads. Aquat Toxicol 101:447–458

Molina-Molina JM, Hillenweck A, Jouanin I, Zalko D, Cravedi J-P, Fernández M-F, Pillon A, Nicolas J-C, Olea N, Balaguer P (2006) Steroid receptor profiling of vinclozolin and its primary metabolites. Toxicol Appl Pharma 216:44–54

Nakamura A, Takanobu H, Tamura I, Yamamuro M, Iguchi T, Tatarazako N (2014) Verification of responses of Japanese medaka (*Oryzias latipes)* to anti-androgens, vinclozolin and flutamide, in short-term assays. J Appl Toxicol 34:545–553

Navarro S, Barba A, Oliva J, Navarro G, Pardo F (1999) Evolution of residual levels of six pesticides during elaboration of red wines. Effect of wine-making procedures in their disappearance. J Agric Food Chem 47:264–270

Nichols HW (1973) Growth media-freshwater. In: Stein JR (ed) Handbook of phycological methods. Cambridge University Press, Cambridge

Oskam G, Van Genderen J, Hopman R, Noij THM, Noordsij A, Piuker LM (1993) A general view of the problem, with special references to the Dutch situation. Water Supply 11:1–17

Otha T, Tokushima S, Shiga Y, Hanazato T, Yamagata H (1998) An assay system for detecting environmental toxicants with cultured cladoceran eggs in vitro: malformations induced by ethylenethiourea. Environ Res A 77:43–48

Papadopoulou-Marukidou E (1991) Postharvest-applied agrochemicals and their residues in fresh fruits and vegetables. J Assoc Off Anal Chem 74:745–765

Perez-Legaspi IA, Rico-Martínez R (1998) Effect of temperature and food concentration in two species of littoral rotifers. Hydrobiologia 387(388):341–348

Preston BL, Snell TW, Robertson TL, Dingmann BJ (2000) Use of freshwater rotifer *Brachionus calyciflorus* in screening assay for potential endocrine disruptors. Environ Toxicol Chem 19:2923–2928

Rico-Martínez R, Snell TW (1997) Mating behavior in eight rotifer species: using cross-mating test to study species boundaries. Hydrobiologia 356:165–173

Schneider S, Kaufmann W, Biesen R, Ravenzwaay B (2008) Vinclozolin—the lack of a transgenerational effect after oral maternal exposure during organogenesis. Reprod Toxicol 25:352–360

Shurin JB, Dodson SI (1997) Sublethal toxic effects of cyanobacteria and nonylphenol on environmental sex determination and development in Daphnia. Environ Toxicol Chem 16:1269–1276

Siebers J, Gottschild D, Nolting HG (1994) Pesticides in precipitation in northern Germany. Chemosphere 28:1559–1570

Skinner MK, Manikkam M, Guerrero-Bosagna C (2011) Epigenetic transgenerational actions of endocrine disruptors. Reprod Toxicol 31:337–343

Snell TW (2011) A review of the molecular mechanisms of monogonont rotifer reproduction. Hydrobiologia 662:89–97

Snell TW, DesRosiers NJD (2008) Effect of progesterone on sexual reproduction of *Brachionus manjavacas* (Rotifera). J Exp Marine Biol Ecol 363:104–109

Snell TW, Moffat BD (1992) A 2-d life cycle test with the rotifer *Brachionus calyciflorus*. Environ Toxicol Chem 11:1249–1257

Spencer EY (1964) Guide to the chemical used in crop protection. J Chem Soc 4:228–229

Stebbing EP, La Clair JJ (1982) Hormesis—the stimulation of growth by low levels of inhibitors. Sci Total Environ 22:213–234

Stout EP, La Clair JJ, Snell TW, Shearer TL, Kubanek J (2010) Conservation of progesterone hormone function in invertebrate reproduction. Proc Natl Acad Sci USA 107:11859–11864

Thoma K, Nicholson BC (1989) Pesticide losses in runoff from horticultural catchment in South Australia and their relevance to stream and reservoir water quality. Environ Technol Lett 10:117–129

Tillmann M, Schulte-Oehlmann U, Duft M, Markert B, Oehlmann J (2001) Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part III: cyproterone acetate and vinclozolin as antiandrogens. Ecotoxicology 10:373–388

US EPA (2003) Vinclozolin: notice of filing a pesticide petition to establish a tolerance for a certain pesticide chemical in or on food, Federal Register. http://www.gpo.gov/fdsys/pkg/FR-2003-03-26/pdf/03-7246.pdf. Accesed 29 Jan 2015

USEPA (2000) Reregistration eligibility decision for vinclozolin. Prevention, pesticides and toxic substances. http://www.epa.gov/opp00001/reregistration/REDs/2740red.pdf. Accessed 29 Jan 2015

USEPA (2001) National toxicology programś report of the endocrine disruptors low dose. National Institute of Environmental Health Sciences, NIH, National Toxicology Program. http://ntp.niehs.nih.gov/ntp/htdocs/liason/lowdosepeerfinalrpt.pdf. Accessed 29 Jan 2015

Uzumcu M, Suzuki H, Skinner MK (2004) Effect of the anti-androgenic endocrine and function. Reprod Toxicol 18:765–774

Vandegehuchte MB, Lemière F, Vanhaecke L, Vanden Berghe W, Janssen CR (2010) Direct and transgenerational impact on *Daphnia magna* of chemical with a known effect on DNA methylation. Comp Biochem Physiol C 151:218–285

Wallace RL, Snell TW, Ricci C, Nogrady T (2006) Rotifera: biology, ecology and systematics. In: Dumont HJF (ed) Guides to the identification of the microinvertebrates of the continental waters of the world. Kenobi Productions, Backhuys Publishers, Leiden

Watermann BT, Gnass K, Kolodzey H, Thomsen AE (2011) Laboratory tests with androgenic and antiandrogenic pesticides—comparative studies on endocrine modulation in the reproductive system of invertebrates and vertebrates. intechopen. http://www.intechopen.com/books/pesticides-in-the-modern-world-effects-of-pesticides-exposure/laboratory-tests-with-androgenic-and-anti-androgenic-pesticides-comparative-studies-on-endocrine-mod. Accessed 29 Jan 2015

Welshons WV, Nagel SC, Thayer KA, Judy BM, Vom Saal FS (1999) Low-dose bioactivity of xenoestrogens in animals: fetal exposure to low doses of methoxychlor and other xenoestrogens increases adult prostate size in mice. Toxicol Ind Health 15:12–25

Yang L, Jinmiao Z, Li W, Li Z, Wang Z (2011) Vinclozolin affects the interrenal system of the rare minnow (*Gobiocypris rarus*). Aquat Toxicol 104:153–159

Zavala-Aguirre JL, Torres-Bugarin O, Zamora-Perez AL (2007) Aquatic ecotoxicology approaches in Western Mexico. J Environ Sci Health A Tox Hazard Subst Environ Eng 42:1503–1511

Zou E (2003) Current status of environmental endocrine disruption in selected aquatic invertebrates. Acta Zool Sin 49:551–565