J Soils Sediments (2009) 9:492–500
DOI 10.1007/s11368-009-0104-y

SOILS, SEC 4 • ECOTOXICOLOGY • RESEARCH ARTICLE

Endocrine disruption in a terrestrial isopod under exposure to bisphenol A and vinclozolin

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Received: 21 January 2009 / Accepted: 17 April 2009 / Published online: 2 July 2009

Abstract
Background, aim, and scope In the past decade there has been an increasing awareness about the possible consequences of human and wildlife exposure to endocrine disrupting compounds (EDCs). Bisphenol A (BPA) and vinclozolin (Vz) are EDCs which impacts on vertebrates have been largely investigated. Nevertheless, research on invertebrate effects, especially on soil organisms, are still largely underrepresented. This work aims to extend the limited ecotoxicological datasets available and to provide tools to assess the effects of EDCs on the terrestrial species, using *Porcellio scaber* (Crustacea: Isopoda) as a model organism.
Materials and methods Male adult isopods were exposed for 10 weeks to BPA and Ronilan® [containing 50% Vz as active ingredient (a.i.)] at concentrations of 10, 30, 100, 300, and 1,000 mg a.i./kg of soil and compared to non-exposed isopods. We studied the effects of these EDCs on molting and total ecdysteroid (20E) concentration. Young, sexually undifferentiated isopods were also exposed to these compounds (Vz, 5, 10, 25, 50, and 100 mg a.i./kg of soil; and BPA, 10, 25, 50, 150, and 300 mg/kg of soil) for 16 weeks and effects on sex ratio were assessed.
Results Exposure to both chemicals resulted in toxic effects on isopods. Time to first molt was delayed with increasing concentrations of Vz. After 10 weeks exposure to 1,000 mg a.i. Vz/kg soil, 100% mortality occurred due to incomplete ecdysis. BPA induced an opposite effect as animals started to molt sooner. Vz significantly increased the 20E titres after 7 and 14 days (LOEC 300 mg a.i. Vz/kg soil) and after 28 days of exposure the LOEC value was 100 mg a.i. Vz/kg soil. BPA also induced a 20E concentration increase after 28 days of exposure at 10, 300, and 1,000 mg/kg soil. In juveniles, we observed a low-dose alteration of sex ratio in BPA-exposed organisms with a skewed ratio of one male per two females, which is in contrast to an almost equal gender distribution in the control. Vz induced no alterations in the sex ratio of isopods.
Discussion Results show that chronic Vz exposure induces a high mortality in *P. scaber*. This is not consistent with other studies describing non-toxicity of fungicides to arthropods. Therefore, it is desirable that toxicity assessment of fungicides is performed via chronic exposure and full life cycle tests. Previously reported low-dose responses to BPA in vertebrates are consistent with results of the present study regarding a sex-ratio shift induced by low BPA concentrations. Enhanced mortality turned out to be the effect of incomplete ecdysis related to increased ecdysteroids titres. Therefore, 'hyperecdysonism' might be a promising endpoint to detect and assess endocrine disruption (ED) in arthropods inhabiting the terrestrial environment.
Conclusions This work reveals that both Vz and BPA disrupt the endocrine function of these important representatives of soil edaphic invertebrates. For the first time, the existence of 'low-dose effects' affecting soil invertebrates is reported. Therefore, isopods are suitable organisms for ED assessment and endpoints such as molting, sex ratio, or 20E

Responsible editor: Jörg Römbke

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concentration are valuable tools for ecotoxicological studies on hormonally active substances.

Recommendations and perspectives Although the effects observed in the present study have not been induced at environmentally relevant concentrations, synergistic interactions of EDC mixtures present in the environment may well have an impact on arthropods at lower substance concentrations. Additionally, the low-dose sex-ratio change demonstrated here confirms the importance of the choice for a large concentration range. The assessment of potential EDCs should consider chronic exposures and life cycle studies. Although the modes of action of EDCs in many arthropods are fragmentary, parameters like molting impairment, incomplete ecdysis, and the determination of hormone titres seem to be suitable biomarkers that should be included as soon as possible in regular surveys for the detection of hormonally active substances.

Keywords 20-hydroxyecdysone · Bisphenol A · Endocrine disruption · Ecotoxicological testing · Hyperecdysonism · Incomplete ecdysis · Low-dose effects · Molting · *Porcellio scaber* · Sex ratio · Terrestrial isopods · Vinclozolin

1 Background, aim, and scope

There has been a growing concern about the possible consequences of wildlife and human exposure to xenobiotic compounds which are able to modulate the endocrine system, the endocrine disruptor compounds (EDCs). Meanwhile, this issue has also been addressed via several priority research actions and legislation by the European Union (European Commission 2007), the US Environmental Protection Agency (Harding et al. 2006), and the World Health Organization (Damstra et al. 2002).

Around 95% of known species in the animal kingdom are invertebrates (deFur et al. 1999). Nevertheless, studies on the effects of EDCs on invertebrates are still scarce and, of these, only 10% were conducted with terrestrial invertebrates (Oehlmann and Schulte-Oehlmann 2003).

Processes under endocrine control, like the molting of arthropods (insects, crustaceans, and of some other minor groups), require periodic loss of the exoskeleton, replacement and hardening with a larger new cuticle. Molting is regulated by ecdysone and related compounds called ecdysteroids (Horn et al. 1966). With the decline of 20-hydroxyecdysone (20E) titres, the hydroxylated and biologically active ecdysteroid, the production of a new cuticle begins. When a threshold value of the ecdysteroid titre is attained, a series of neuropeptides are released causing shedding of the old cuticle by a process termed ecdysis (Subramoniam 2000). Thus, while classical toxicity testing methodologies are not designed to specifically address ED (e.g., survival, sex ratio, growth rate, molting intervals, and success), they can measure adverse integrative effects associated with EDCs.

Isopods are saprophytic organisms that play a key role in the organic matter decomposition in soils. Their importance in the soil system, allied to their easy maintenance in laboratory cultures, sexual reproduction, and dimorphism, size of organisms and their distinct molting stages, make isopods ideal model test organisms for ecotoxicological studies (Drobne 1997). Isopods have been studied after exposure to a wide range of toxicants, including heavy metals (e.g. Loureiro et al. 2009) and organic compounds (e.g. Engenheiro et al. 2005). Most information about the effects of EDCs is available essentially at the toxicological level. At the environmental level the information is scarcer and focused on the effects involving aquatic organisms, thus creating a gap for the soil system. Therefore, this work aims to extend data on the effects of EDCs on the terrestrial species, using *Porcellio scaber* (Crustacea, Isopoda) as a model organism.

In this study, two compounds, listed under the EU priority list of ED (European Commission 2001), were investigated. Vinclozolin (Vz, 3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione; CAS 50471-44-8) is a fungicide which was widely used in agriculture for the production of lettuce, raspberries, beans, onions, vines, and others. In Europe, the use of this substance is no longer authorized according to the exclusion of Vz from Annex 1 under EC Directive 91/414. It is a proven endocrine disruptor causing anti-androgenic effects due to its metabolites, which are able to bind to the androgen receptor (Kelce et al. 1994). Vz induces a reduction of the penis and accessory male sex organs and advancement of the sexual response phase in prosobranch snails (Tillmann et al. 2001) and shows epigenetic transgenerational effects (Anway et al. 2006). According to our knowledge, data on accumulation in soil or effects on soil organisms are not available.

Bisphenol A (BPA, 4,4′-dihydroxy-2,2-diphenylpropane; CAS 80-05-7) has been shown to be an estrogen receptor agonist (Okada et al. 2008) for which anti-androgenic properties were also identified (Lee et al. 2003). It is used to produce epoxy and polycarbonate resins which are employed in the manufacture of a wide range of consumer products, such as food containers, and in medical applications. According to Euling and Sonawane (2005) about 3,200,000 tonnes of BPA are manufactured worldwide each year (based on data for 2005). The presence of BPA in sewage sludge from municipal plants [concentrations of 0.033–36.7 mg/kg (dw)] (Lee and Peart 2000) and its application to the land to function as soil improver

(Furhacker et al. 2000) indicate that contamination of agricultural soils is a real issue, although neither toxicity data for soil organisms nor soil residue data are available.

2 Materials and methods

2.1 Test organism and culture procedures

Isopods (*P. scaber*, Latreille 1804) came from a culture established in our laboratory for more than 8 years, initiated with animals collected from horse manure in central Portugal. Animals in culture are maintained at 21±1°C, with a 16:8 (light–dark) photoperiod in plastic boxes with a layer of sterile sand and food provided ad libitum in the form of oven-dried alder (*Alnus glutinosa*) leaves and with 100% relative air humidity.

Pregnant females, with fully formed marsupium, were placed in a secondary culture until release of mancae. Isopods born within a 2-day period were used to start synchronized cultures and later reared individually in polyethylene terephthalate (PET) boxes (Ø60×30 mm) perforated on the sides to insure ventilation and applying the same substrate and conditions described above.

2.2 Chemicals and preparation of soil

An agricultural natural soil from the lower Mondego valley (Portugal) kept in fallow and with no intervention or plant protection products use for the last 5 years was used in these experiments. The soil was oven dried at 60°C for 48 h and immediately weighed. Both contaminations were carried out on dry soil.

Bisphenol A (Merck Schuchardt, Germany, purity >99%) was dissolved in equal amounts of methanol and mixed with soil at 10, 30, 100, 300, and 1,000 mg/kg dry soil for experiments with adults. For juvenile testing, toxicant concentrations were 10, 25, 50, 100, and 300 mg/kg dry soil. After BPA spiking, soil was left to dry under a fume hood for 12 h. Moisture content was subsequently adjusted to 20% (*v*/*w*) with distilled water. As a control, moisture content was adjusted as described above. A solvent control (0^+) was prepared with the same volume of methanol without BPA, then left to dry and moisture content adjusted as described above.

Vinclozolin (Ronilan® containing 50% Vz as active ingredient (a.i.); BASF AG, Germany) dissolved in water, was mixed with soil at 10, 30, 100, 300, and 1,000 mg a.i./kg dry soil for adult experiments and at 5, 10, 25, 50, and 100 mg a.i./kg dry soil for juvenile testing. The soil moisture content was then adjusted to 20% (*v*/*w*) with distilled water.

Chemical analysis of Vz and BPA treatments concentration were performed and were within ±5% of the nominal concentrations. The results are presented in terms of the nominal values.

2.3 Organism exposure

2.3.1 Adult isopod testing (molting and 20-hydroxyecdysone)

All experiments were performed at the same temperature, photoperiod, and relative humidity conditions as described above for *P. scaber* cultures. Isopods were taken from the lab culture weighing 20±1 mg. Only male adults were used to ensure that female reproductive traits did not influence the endpoints assessed.

Thirty-two animals, at intermolt stage, per treatment were randomly and individually placed in PET boxes (Ø100×50 mm) filled with 60 g of spiked agricultural soil and four Ø10-mm alder leaf disks providing the same shelter area and food. Leaf disks were added weekly, when necessary, in order to maintain food ad libitum. Molting stage was determined at weekly intervals for 10 weeks.

2.3.2 Juvenile isopod testing (sex ratio determination)

Fifty juveniles per treatment, weighing between 4 and 5 mg, were separated before secondary sexual characters occurred, insuring also virginity of the animals and, thus, preventing reproductive processes during the experimental period. These animals were randomly and individually placed in PET boxes (Ø60×30 mm) filled with 30 g of spiked agricultural soil and two Ø10-mm alder leaf disks, which provided the same shelter area and food. Leaf disks were added weekly, when necessary, in order to maintain food ad libitum. All juvenile isopods were exposed to toxicants before differentiation of the secondary sexual characters. After the 16-week experimental period, sex was determined, and the sex ratio of test organisms was calculated for each treatment group. Gender was determined by checking male differentiation of the endopodites on the second pair of pleopods formed into copulatory legs.

2.4 Measurements, photographs, and image analysis

P. scaber, as all crustaceans, has saltatory growth, increasing size after every ecdysis. An increase of cuticle size is a reliable sign that they have molted. This, together with the verification of calcium deposits on thoracic sternites (Zidar et al. 1998), guarantees the reliable identification of molting occurrence. The medium segment of 12 adults per treatment was photographed under stereo dissecting microscope for

posterior digital image analysis using Leica Qwin, Image Processing and Analysis Software® for cephalothorax width measurements. Occurrence of molt was inferred from the difference of size in consecutive measurements.

2.5 Ecdysteroid analysis

After 1, 2, and 4 weeks, eight intermolt individuals per treatment were analyzed for ecdysteroid quantification. Isopods were frozen in liquid N_2, lyophilized (Snijders Scientific, type 2040 lyophilizer, Tilburg, The Netherlands) and dry weights were measured. Afterwards, individuals were homogenized in methanol and prepared according to the method of Block et al. (2003). Ecdysteroid measurements were carried out with 20-hydroxyecdysone EIA kits from Cayman Chemical Company (Ann Arbor, MI, USA) according to the manufacturer's instructions. The colorimetric reaction was measured at 414 nm using the Labsystems Multiskan EX plate reader (Helsinki, Finland). Ecdysteroid titre of each sample was determined by comparison of sample absorbances with the 20-hydroxyecdysone standard curve and expressed as pg of ecdysone equivalents/mg dw (Polgar et al. 1996).

2.6 Statistical analysis

LC50 values and corresponding 95% confidence limits were determined by the probit analysis method (Finney 1971). All data were checked for normality and homoscedasticity. One-way analysis of variance (ANOVA) with Dunnett's multiple comparison of group means were employed to determine significant differences relatively to control treatment. Significant differences for sex ratios relatively to control treatment were assessed using the *G*-test for goodness-of-fit for which two-tailed p values were calculated. Where applicable, results are presented as mean±SE. For all statistical tests, the significance level was set at $p \leq 0.05$. All calculations were performed with SigmaStat (Systat Software Inc. 2006).

3 Results

3.1 Lethality of vinclozolin and bisphenol A

After 4 weeks exposure of adult *P. scaber*, LC_{50} values were higher than 1,000 mg a.i. Vz and BPA/kg soil (37.5% mortality at this highest concentration for both test chemicals). After 10 weeks of exposure, 1,000 mg BPA/kg soil killed 50% of the animals [LC_{50} (95% CI) was 910 (163–1,658) mg/kg soil]. Exposure to 1,000 mg a.i. Vz/kg soil led to 100% mortality and half of the adult isopods died at 300 mg a.i. Vz/kg soil, resulting in an LC_{50} (95% CI) of 298 (150–447) mg/kg soil. The LC_{50}s for the juvenile isopods were higher than the highest concentrations tested for both chemicals, even after 16 weeks.

3.2 Developmental toxicity of vinclozolin and bisphenol A

The majority of organisms in control conditions molted in the first 2 weeks, 25% in week 1 and 50% in week 2. About 87.5% of isopods exposed to Vz (10 mg a.i. Vz/kg soil) molted in the first 2 weeks (Fig. 1a). Molt seemed to be drastically delayed with increasing concentration of the contaminant. In the highest concentration tested, 1,000 mg a.i. Vz/kg soil, only 12.5% of animals molted in the first 2 weeks, 50% of them only molted in the second month.

Isopods kept in soil treated with the BPA's solvent, methanol, experienced an extremely delayed molt with 87.5% of first molts being observed only in the third week (Fig. 1b). This severe molt delay as a consequence of solvent exposure seems to be mitigated by increasing levels

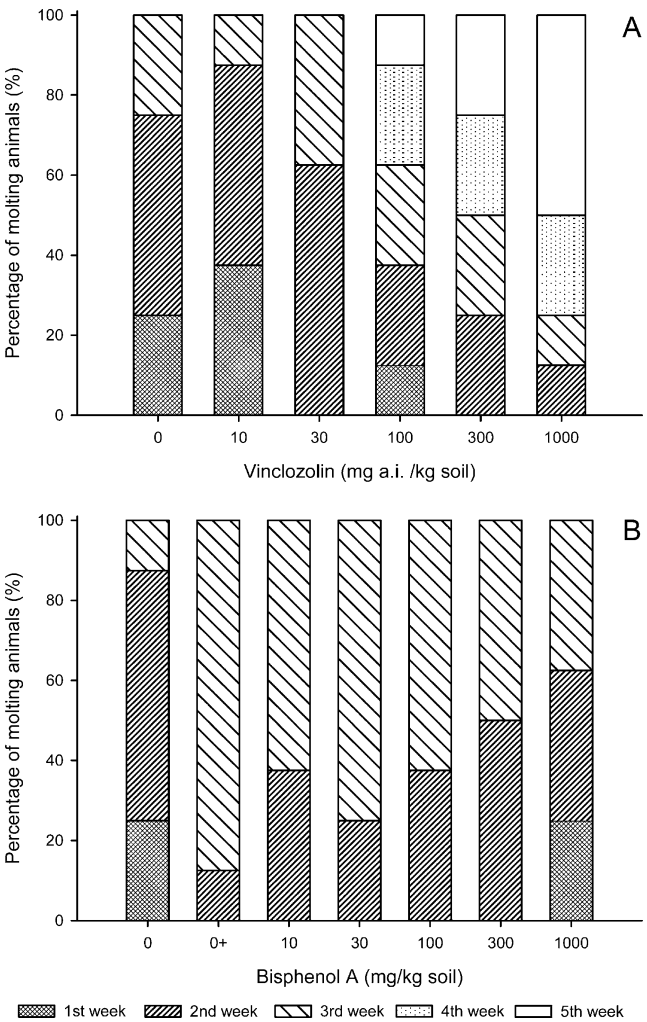

Fig. 1 Distribution of first molt per week of *Porcellio scaber* after exposure to soil treated with **a** vinclozolin and **b** bisphenol A. Data are presented as percentage of animals that molted during each period

of BPA in a concentration-dependent way. At the BPA concentration of 1,000 mg/kg soil a quarter of the animals molted in the first week and around 63% in the first 14 days, resembling the control conditions.

3.3 Ecdysteroids titres

The levels of 20E during the intermolt period were determined for adult male isopods. An average value of 29.7±4.3 pg ecdysone equivalents/mg dw during intermolt reaching a peak concentration of 79.4±3.8 pg ecdysone equivalents/mg dw during premolt was observed for the control group. Vz induced a time and concentration-dependent increase of 20E concentration (Fig. 2a). The level of 20E increased after 7 and 14 days exposure with a NOEC of 100 mg a.i. Vz/kg soil (ANOVA, Dunnett's test, $F_{5,39}$=10.553, p<0.001 and $F_{5,38}$=5.757, p<0.001, respectively). Furthermore, after 28 days of exposure to the fungicide, 20E titres were elevated compared to controls, being statistically significant from 100 mg a.i. Vz/kg soil onwards (ANOVA, Dunnett's test, $F_{5,38}$=6.925, p<0.001).

In BPA contaminated soil (Fig. 2b), although ANOVA detected differences of 20E titres of 14 days exposed groups compared to solvent control (ANOVA, Dunnett's test, $F_{5,35}$=4.752, p<0.001), the post hoc test was not able to distinguish any differences. With extended exposure (28 days), 20E titres increased and were significantly different for animals exposed to the lowest (10 mg BPA/kg soil) and the highest concentrations (300 and 1,000 mg BPA/kg soil), when compared to the solvent control (ANOVA, Dunnett's test, $F_{6,39}$=5.270, p<0.001).

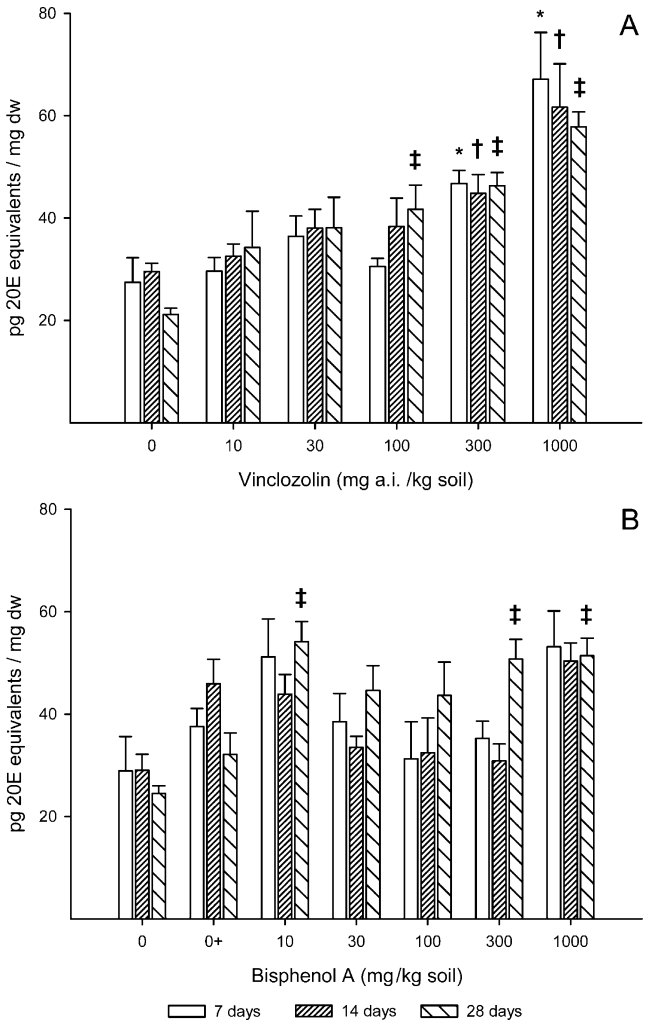

Fig. 2 Ecdysteroid titres of *Porcellio scaber* following 7, 14, and 28 days exposure to **a** vinclozolin or **b** bisphenol A. Significant differences from control or solvent control (p<0.05, ANOVA, Dunnett's test): *7 days; † 14 days; and ‡ 28 days

3.4 Gender ratio in isopods exposed to vinclozolin and bisphenol A

The sex ratio found under control conditions (number of males/number of females) was 0.95, i.e., nearly one male per every female (Fig. 3). Exposure to the fungicide Vz did not alter this ratio (Fig. 3a). A skewed sex ratio was observed for BPA (Fig. 3b). Gender ratio in the lowest concentration (10 mg BPA/kg soil) changed in favor of females to a ratio of one male per two females (G test, G= 5.303, df 1, p=0.021). Higher BPA concentrations in the soil resulted in the same effect, although differences were not statistically significant.

4 Discussion

To date, no toxicity data of Vz and BPA has been presented for isopods. Vz and BPA revealed very low mortality rates for the 28 days adult exposure test. When extending the exposure period to 10 weeks we found a severe increase of mortality in Vz-exposed treatment groups. This is not consistent with the common low acute toxicity to arthropods shown for other fungicides (Jansch et al. 2005; Haeba et al. 2008). Vinclozolin has a low to moderate persistence in soil (IUPAC 2006), with reported half-lives from 28–43 days in the laboratory up to 34–94 days in the field (US EPA 1991; IUPAC 2006). Despite the low-persistency of Vz, this increase of mortality with exposure time may be due to the increased concentration of its major metabolites, M1, M2, and 3,5-DCA, which are reported to be more toxic than the parent compound (Kelce et al. 1994) and with half-lives ranging from 179 to >1,000 days (US EPA 2000). Therefore, it is desirable that toxicity assessment of fungicides is performed via chronic exposure and full life cycle tests.

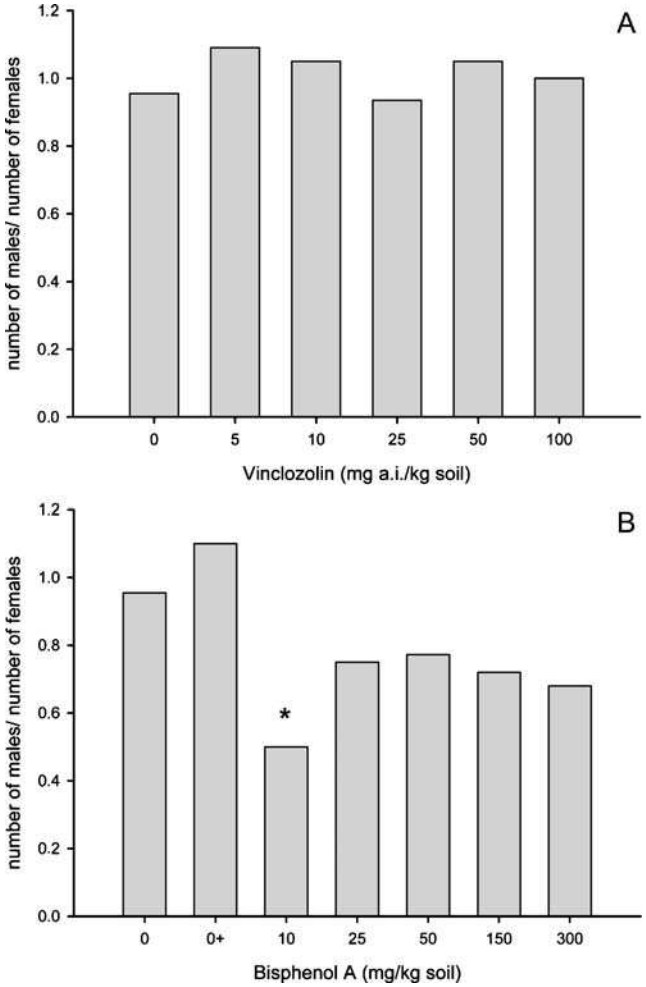

Fig. 3 Sex ratio of *Porcellio scaber* after exposure to soil treated with **a** vinclozolin and **b** bisphenol A, represented as the number of males/ number of females. An *asterisk* indicates a significant difference from the solvent control ($p \leq 0.05$, *G*-test)

Molting has been considered as an important endpoint by several authors and it is known to be affected by various substances and conditions, such as temperature and nutritional state of the organism (Weis et al. 1987; Drobne and Strus 1996). Although development and molting parameters cannot be considered exclusive in the assessment of hormonally active substances, they can measure adverse integrative effects associated with ED when a direct causal link with hormonal disruption is proven (deFur et al. 1999).

The solvent used in the experiments, methanol, introduced a large difference in the time to reach molt, probably by altering the physical properties of the soil (water holding capacity, organic matter availability, and others), thereby creating stressful situations that have affected the molt process. Nevertheless, there was a clear reduction in the time taken to first molt caused by the exposure to BPA when compared to the solvent control treatment.

The xenoestrogen BPA induced a sex-ratio shift favoring female isopods. In prosobranch snails, it has been reported that female-biased sex ratios were caused by the phenomenon of feminization induced by BPA (Oehlmann et al. 2000). Nevertheless, the parsimony principle withstands the simpler and straightforward explanation that a skewed sex ratio could simply be due to differential lethality of one of the genders to the toxicant (Callahan and Weis 1983). This issue could be tackled in the future by performing gonad histopathology in exposed isopods and assessing abnormalities associated with masculinized females or feminized males, or by identifying genetic sex markers that can be used to compare genetic sex with individual phenotypic secondary sexual characters.

Although other than endocrine modes of action cannot be excluded, the classical concentration response paradigm was replaced by a sex-ratio shift and increased molting hormone levels at low concentrations, which is in agreement with several studies where lower concentrations of BPA induced larger effects (Welshons et al. 2006; Izumi et al. 2008). Furthermore, this is backed by the US EPA expert panel which confirms these exclusively low-concentration effects of endocrine-mimicking chemicals (Kaiser 2000). In a *Drosophila melanogaster* B_{II} cell in vitro assay, BPA was able to compete with ecdysteroids for the ligand binding site on the receptor complex (Dinan et al. 2001). Planello et al. (2008) demonstrated that BPA acts as an ecdysone-mimetic compound and up-regulates the levels of ecdysone receptor (EcR) in *Chironomus riparius* (midge) cells. This competition for the receptor might be responsible for the 170% increase of the 20E levels in the lowest concentration tested (10 mg BPA/kg soil) and over 148% increase for the two highest concentrations (300 and 1,000 mg BPA/kg soil) after 28 days of exposure. The reason for this hormone balance impairment remains speculative and, as argued by Mu et al. (2005) and supported by the current work, the precise mechanism of toxicity does not involve depletion of endogenous ecdysteroid levels. Nonetheless, one may hypothesize that ecdysone receptor binding feedback mechanisms might not have been triggered due to unspecific binding and that production/elimination mechanisms might have been unbalanced leading to increased 20E titres. Vz, in contrast to BPA, delayed the first molt, with highest mortality observed only at higher concentrations as a consequence of incomplete ecdysis during this first molt. Weis et al. (1987) also noted significant mortality close to the time of ecdysis at higher concentrations of diflubenzuron in the fiddler crab *Uca pugilator* and Baldwin et al. (2001) observed mortality at this molting stage in *Daphnia magna* exposed to 20E and ponasterone A.

Prior to ecdysis, ecdysteroid titres raise and a sudden decrease of these levels trigger the completion of exuviation

(Subramoniam 2000). It has been reported that the phenomenon of hyperecdysonism—a higher basal level of 20E—overrides this normal precipitation of ecdysteroids preventing animals from exuviating probably by impairing the release of an exuviation factor (Bodar et al. 1990). In our study, the mortality due to incomplete ecdysis may be related to the high levels of ecdysteroids found with increasing concentrations of Vz. Due to the lack of exuviation factor animals suffered from delayed or incomplete molts, cumulating in mortality at higher substance concentrations. Therefore, incomplete ecdysis and hyperecdysonism may become potential bioindicators to detect disruption of ecdysteroid function.

Vz is a proposed anti-androgen that has been shown to interfere with steroid hormone homeostasis. Hormonal balance plays a decisive role in both primary sexual determinations and acquisition and maintenance of secondary sex characteristics in adults, thus establishing gender during the pre- and neonatal period (Colborn et al. 1993; LeBlanc et al. 1997). Signs of demasculinization and female-biased populations were confirmed by several authors. Haeba et al. (2008) found a twofold decrease in the number of male neonates in *D. magna* exposed to 1 mg Vz/L and in molluscs exposed to Vz, where the lengths of the penis and of accessory male sex organs (e.g. penis sheat, prostate) were reduced (Tillmann et al. 2001). Nevertheless, in our experiments, Vz exposure had no significant effects on the gender ratio in isopods.

EDCs acting at the level and time of sexual differentiation in an invertebrate may skew sex ratios in favor of males (Matthiessen and Gibbs 1998; Olmstead and LeBlanc 2003) or females (Oehlmann et al. 2000; Haeba et al. 2008) depending on the estrogenic or androgenic mechanisms involved. The study of sex ratios of natural populations might provide a simple and robust indication of EDC activities in the environment provided that the normal gender distribution of undisturbed populations is known. Also, the characteristic patterns of ecdysteroid concentration in isopods during the molt cycle provide a measurable marker of hormonal function. As soon as baseline data have been established for control populations, it is possible that measurements of changes in ecdysteroid titres can be applied as a routine tool for biomonitoring studies, including the detection of hormonally active substances.

5 Conclusions

Vinclozolin and bisphenol A are well-documented endocrine disruptors in vertebrates and have already been investigated in some aquatic invertebrates. The results from the present study demonstrate that both the fungicide vinclozolin and the industrial chemical bisphenol A cause endocrine disruption in *P. scaber* with an ecdysteroid up-regulation resulting in molting disturbances. Since endocrine-mediated chronic effects were identified at much lower concentrations than those showing acute toxic effects on isopods, molting impairment, incomplete ecdysis, and hormone levels are easy and suitable biomarkers that may provide further valuable endpoints for EDCs identification and characterization.

6 Recommendations and perspectives

Isopods like *P. scaber* combine the features of continuous growth, through a molting regime, with a terrestrial mode of life, making them suitable sentinel species to detect hormonally active soil pollutants. After the maximum recommended application rate of Ronilan®, the concentration of vinclozolin in the soil is 1 mg a.i./kg (assuming that 70% of the fungicide will reach the surface and is homogeneously distributed over the top 5-cm soil layer and the soil bulk density is 1.4 kg/dm^3). Vinclozolin has a low to moderate persistence in soil, with reported half-lives from 28–43 days in the laboratory up to 34–94 days in the field (US EPA 1991; IUPAC 2006). The only significant route of BPA to the terrestrial environment is through the application of sewage sludge from municipal plants [concentrations of 0.033–36.7 mg/kg (dw)] (Lee and Peart 2000) as soil improvers. The half-life for bisphenol A in soil was calculated as from 3 days (Fent et al. 2003) up to 37.5 days (based on modeled half-life in water) (Environment Canada 2008). Despite the concentrations resulting in adverse effects in the present study are well above these expected environmental levels and, at a first glance, it seems that vinclozolin and bisphenol A pose no threat to natural populations of terrestrial arthropods, caution should be taken in ecological risk assessments since chemicals that interfere with ecdysteroidal activity are common in the environment, and effects due to this class of compounds might become more enhanced in mixtures than at individual concentrations previously classified as safe.

Acknowledgments M.F.L. Lemos was supported by Fundação para a Ciência e Tecnologia (SFRH/BD/13868/2003). Parts of this work were supported by FCT Project Grant reference PTDC/BIA-BDE/75690/2006.

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