Ecotoxicology (2016) 25:105–114
DOI 10.1007/s10646-015-1571-0

Estrogenic contamination by manure fertilizer in organic farming: a case study with the lizard *Podarcis sicula*

Mariailaria Verderame[1] · Ermelinda Limatola[1] · Rosaria Scudiero[1]

Accepted: 9 October 2015 / Published online: 16 October 2015

Abstract In the last years, worldwide organic farming has grown exponentially; as a consequence, the use of animal manure as a soil fertility source has become the principal agricultural choice. However, the use of manure as fertilizer can increase the amount of steroid hormone metabolites in the soil. In southern Italy, lacertidae lizards are the most abundant vertebrate group in agroecosystems and have been identified as potential model species for ecotoxicological studies. The aim of this study was to understand if the manure applied in organic farming has estrogen-like effects in the lizard *Podarcis sicula.* Adult male lizards were captured in two organic agricultural fields (manure-treated sites) and in an uncultivated field (control site). Lizards from the two organic farms displayed hepatic biosynthetic alterations typical of an estrogenic contamination; hepatocytes contained both vitellogenin and estrogen receptor alpha transcripts and proteins, detected by in situ hybridization and immunocytochemistry. The same cells did not show cadmium, lead and metallothionein accumulation, indicative of the lack of inorganic contamination. These findings suggest that exogenous estrogens, arising from the use of manure, could affect the welfare of wild animals and animal breeding, leading to bioaccumulation of estrogens in food chain, with possible risk for human consumers. For this reason, organic farming should implement the use of sustainable practices such as crop rotation to preserve the soil biological activity, rather than organic manure as fertilizer.

✉ Rosaria Scudiero
rosaria.scudiero@unina.it

[1] Department of Biology, University Federico II, Via Mezzocannone 8, 80134 Naples, Italy

Keywords Estrogen receptor alpha · Heavy metals · Manure · Metallothionein · Organic farming · Vitellogenin

Introduction

Increasingly emerging data on the effects of chemical fertilizers and pesticides to human health and environmental impact led in recent years to the sharp increase in agricultural practices and animal husbandry of organic crops and livestock. The organic farming allows the use of many natural derivates as fertilizers [Council Regulation (EC) No 834/2007 and Commission Regulation (EC) No 889/2008]. Fertilizers including animal and vegetable derivates can display estrogen-like activity and are able to bind and activate steroid receptors (Degen and Bolt 2000; Combalbert et al. 2012; Alvarez et al. 2013; Valdehita et al. 2014); some phytosanitary products, as well as products for disinfection and cleaning in the livestock and crop production, have been classified as endocrine disruptor chemicals (EDC) (Ying et al. 2002). Fertilizer of animal origin is made of bones, slaughter residues and manure that may contain residues of hormonal substances and/or their metabolites (Bartelt-Hunt et al. 2013; Valdehita et al. 2014). Indeed, vertebrates excrete conjugated or free steroid hormones that may interfere with the endocrine system, affecting the reproduction and development in wildlife (Jobling et al. 1996). It has been demonstrated that metabolites of steroid hormones persist in manure for several months (Schiffer et al. 2001). In addition, conjugated and biologically inactive forms are easily converted into free steroids by soil microrganisms (Ternes et al. 1999; Baronti et al. 2000). *Escherichia coli* in activated sludge is able to deconjugate human hormones, excreted in the urine as inactive glucuronide or sulphate

conjugates, in the active steroids (Desbrow et al. 1998). As consequence, when manures are applied to the soil as fertilizer, these compounds may reach living organisms inducing abnormal endocrine response (Combalbert et al. 2012). Recently, bioassay of estrogenicity on water samples from streams across the United States revealed that higher estrogenic activity was frequently associated with manure application to cultivated fields (Alvarez et al. 2013).

The most common responses are activation of estrogen receptors (ERs) and biosynthesis of vitellogenin (VTG), the estrogen-dependent and sex-specific yolk protein naturally occurring only in females of oviparous vertebrates during the reproductive period (Tata and Smith 1979; Wallace 1985). In the males of oviparous species, VTG gene is silent but can be activated by experimental treatment with estradiol or by exposure to xenoestrogens. As a consequence, the presence of VTG in males is considered the most reliable biomarker of estrogen contamination (Denslow et al. 1999; Irwin and Gray 2001; Matozzo et al. 2008; Verderame et al. 2011; Del Giudice et al. 2012).

Manures of both animal and vegetal origin can contribute to heavy metal loads on agricultural soils; heavy metal contents vary considerably between manure types and origins (Menzi et al. 1993). The role of chemical fertilizers and atmospheric deposition on lead (Pb) and cadmium (Cd) accumulation in topsoils is well known (N'guessan et al. 2009), however, manure of animal origin could contain Cd concentrations exceeding those permitted in agricultural soils (Li et al. 2010; Zhou et al. 2015). Cd is a well recognized endocrine disruptor by affecting the synthesis and/or regulation of several hormones (Henson and Chedrese 2004; Darbre 2006) and exerting an estrogen-like activity in vitro and in vivo (Iavicoli et al. 2009; Hofer et al. 2010). Recent studies have shown that in mammals, Cd may also activate the estrogen receptor α (ERα) and/or mimic estrogen effects in different tissues (Johnson et al. 2003). Cadmium is also able to interfere with the estradiol (E2)-controlled gene expression of ERs and VTG genes. In liver of juvenile females of rainbow trout, Cd reduces the E2-stimulated mRNA levels of VTG and ERα in a dose-dependent manner (Vetillard and Bailhache 2005). On the other hand, in *Salmo trutta* exposed to high dose of E2, Cd is able to delay the ovulation but does not inhibit ERs upregulation or modify VTG gene expression (Brown et al. 1994).

Active biomonitoring of soil contamination can use bioindicator organisms such as microbial community, mosses, liverworts and, among terrestrial invertebrates, nematodes and gastropods (Bargagli et al. 1995; Kammenga et al. 2000; Weeks et al. 2004; Caruso et al. 2009; Asensio et al. 2013). But often these organisms do not provide information applicable to more complex systems such as vertebrates nor provide data on the fate of secondary consumers, responsible for controlling the population of many invertebrates as well as flora such as weeds. Lizards are soil surface animals that represent an important link between invertebrates and higher predators. The wall lizard *Podarcis sicula* is the most abundant lizard species present in southern Italy. As prey for many small mammals and birds, it fills an important role also as soil top predator contributing, in several areas, to the control of agricultural pests (Henle and Klaver 1986). Obviously, being part of wild fauna, they can be affected by contamination from anthropic activities. In the lizard *P. sicula*, VTG synthesis in females is accompanied by the expression of ERα (Verderame and Limatola 2010). In males, both VTG and ERα genes are silent; however, it has been demonstrated that both genes can be activated by estrogen stimulation and/or by EDC with estrogen-like activity (Verderame et al. 2011). In addition, EDCs have been shown to interfere with spermatogenesis (Cardone 2012; Verderame and Limatola 2015), as well as Cd ions interfere with the reproductive performance and embryonic development (Simoniello et al. 2011, 2013, 2014).

Considering the increasing consumption of organic products, the main aim of this study was to determine in the liver of *P. sicula* male specimens from organic farmland the presence of VTG and ERα, as good biomarkers of EDC contamination. On the same tissue, it has been also evaluated the concentration of Cd and Pb ions, and the induction of the expression of metallothionein (MT), the principal biomarker of heavy metal contamination (Kammenga et al. 2000; Dallinger et al. 2000, 2004; Trinchella et al. 2006; Simoniello et al. 2010).

Materials and methods

Animals and experimental protocol

Forty-five sexually mature male *P. sicula* (7–8 cm snout-vent length) were caught by noose or hand during their mating period (May–June). Thirty specimens were captured in two organic farms both located in Sorrento Peninsula (Campania, Italy); 15 animals were from a site near Gragnano (BIO-A experimental group) and 15 from a site near Agerola (BIO-B group). The two sites are certified as exclusively organic farms by the Italian Department of Agriculture. Both farms are of wide extent, perched on a hill, far distant and isolated from non-organic crops. These sites use the manure of animals (cows, sheep, horses, pigs and chicken) bred in the same farms as fertilizer. Fifteen animals, referred to as controls (Ctr group), were caught in the outskirts of Naples, in an uncultivated site. The latter represents the wild specimens on which many studies on

the reproductive cycles of this species were performed; they never showed signs of estrogenic contamination (Verderame et al. 2012, 2014; Verderame 2014).

Lizards were kept individually in *terraria* with soil coming from each site and maintained under conditions of natural temperature and photoperiod. Water dishes were always present in the terraria and the animals were fed with uncontaminated larvae of *Tenebrio molitor* and fresh vegetables ad libitum. After 5 days of relaying to remove the effects of capture stress, the animals were anaesthetized by hypothermia chilling in chipped ice and killed by decapitation. Each group was analyzed separately to determine any differences between animals from the two organic farms and between them and the group of animals caught in the uncultivated field. Livers were immediately excised and processed for histological and molecular analyses. Lizards were captured under authorization No.SCN/2D/2000/9213 of the Italian Ministry of the Environment and the experiments were carried out in compliance with the ethical provisions enforced by the European Union and authorized by the National Committee of the Italian Ministry of Health on in vivo experimentation (Department for Veterinary Public Health, Nutrition and Food Safety). Efforts were made to avoid animal suffering and minimize the number of specimens used. Animals were maintained in accordance with the institutional guidelines for care and use of laboratory animals.

Histology

For each animal, small liver pieces (2–3 mm) were fixed in Bouin's fluid, dehydrated in a graded series of alcohols and processed for paraffin embedding. A Reichert-Jung 2030 microtome was used to obtain 7 μm thick sections. Some slides were dewaxed, rehydrated in a graded series of alcohols, rinsed with distilled water and processed for immunocytochemistry (ICC) to detect VTG or MT proteins, and others were processed by in situ hybridization (ISH) to detect VTG, ERα or MT transcripts. For both ICC and ISH analyses, we processed ten slides, each one with four attached sections. All the histological results were examined by using a Nikon-MicroPhot-FXA light microscope.

Immunocytochemistry

The immunoreactive VTG localization was carried out as previously reported (Verderame and Limatola 2010). Briefly, liver sections from the animals of the three different groups were incubated with homologous primary anti-VTG antibody (1:1000) in phosphate buffer (PB) 0.1 M pH 7.4 overnight at 4 °C; washed in the same buffer and incubated with secondary polyclonal biotinylated anti-rabbit antibody (Pierce, Rockford, USA) (1:500 in PB). For ERα localization the primary antibody was an anti-hERα (1:80) (Novocastra, United Kingdom), and the secondary a biotinylated anti-mouse (1:500) (Pierce, USA). To detect antigens, we used the ultrasensitive ABC staining reagent kit (Pierce, USA) and diaminobenzidine (DAB 1 mg/mL; Sigma).

The immunoreactive MT was detect with anti-MT (Thermo Fisher) (1:400) and the antigens were identified with Novolink Max Polymer Detection System (Leica Biosystems) according to the manufacturer's procedure. Negative controls of reactions were performed on other slides by omitting the incubation with the primary antibodies.

In situ hybridization

For in situ hybridization analysis, liver sections mounted onto Superfrost Plus slides (BDH) were dewaxed and treated with proteinase K (10 μg/mL) at 50 °C for 10 min. The *Gallus gallus* VTG (Verderame et al. 2011), *P. sicula* ERα (Verderame and Limatola 2010) and *P. sicula* MT (Riggio et al. 2003) cDNA fragments cloned into the pGEM-T easy plasmid (Promega) were used for generation of cRNA probes. The cRNA synthesis was performed by using the Dig-RNA (SP6/T7) labeling Kit (Roche Applied Science), according to the kit's protocol. After plasmid linearization, the RNA probes were synthesized using RNA polymerase T7 to obtain the sense probes and RNA polymerase SP6 to obtain the anti-sense probes. Digoxigenin (DIG)-labeled anti-sense riboprobes were used at a concentration of 80 ng/100 μL, in hybridization buffer (Tris-HCl 0.02 M, pH 7.5; NaCl 0.3 M; EDTA 0.01 M; DTT 0.1 M; formamide 50 %; Denhardt's 1×; t-RNA 100 μg/mL; ss-DNA 100 μg/mL) overnight at 48 and 50 °C, respectively. The slides were rinsed several times in 2× SSC at room temperature, washed two times in washing buffer (NaCl 0.3 M; formammide 50 %; Tris-HCl 0.02 M; EDTA 0.01 M; DTT 0.1 M) and incubated at 37 °C for 30 min in RNase mix (NaCl 0.5 M; Tris-HCl 0.01 M; EDTA 0.01 M; DTT 0.1 M; RNase 10 μg/mL) and for 30 min in the same mix without RNase. The slides were washed in 2× SSC 3 min, in 0.1× SSC 15 min at 60 °C, in 0.1× SSC 3 min at room temperature, in NTP (Tris-HCl 0,1 M pH 7.5; NaCl 0.15 M) and then incubated 1 h in 2 % blocking solution (Roche Diagnostics, Mannheim, Germany) in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5). The slides were incubated overnight at 4 °C with an alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche Diagnostics) (1:2 500) in blocking solution. The sections were then rinsed four times in NTP buffer for 30 min and two times in NTM buffer (Tris-HCl 100 mM pH 9.5, MgCl 50 mM, NaCl 100 mM) for

30 min. Finally, the sections were incubated in the dark at room temperature in the color detection substrate solution (BCIP/NBT Roche) in NTM until the appearance of the color. The reaction was stopped by rinsing the slides in Tris-HCl 0.1 M pH 7.5 and mounted in Acquovitrex (Carlo Erba). The negative control sections were incubated with the DIG-labeled mRNA sense probes and processed as previously described. Twin sections were treated with DNase to exclude cross-link with genomic DNA.

Determination of cadmium and lead contents in lizard livers

For the determination of total Cd and Pb content in *P. sicula* liver, 0.4 g fresh tissue were digested in concentrated ultrapure HNO_3 (Fluka), using 1 mL of acid every 100 mg of wet tissue. The mixture was heated for 30 min at 80 °C, cooled and centrifuged for 15 min at 12,000×*g*. Cd and Pb content in liver supernatants (n = 15) was determined by Zeeman Graphite Furnace Atomic Absorption Spectrometry, using a Varian A220 atomic spectrophotometer, according to the manufacturer's conditions. Working standards in 0.2 % v/v HNO_3 were prepared daily by diluting known aliquots of the stock solution to the appropriate volume. Metal recovery, calculated as a percentage of known amounts of metal added to the samples, ranged from 90 to 93 % for both metals.

Real time PCR analysis of metallothionein expression

Total RNA was extracted according to the TRI-Reagent (Sigma Aldrich) protocol. The concentration and purity of RNA samples were determined by UV absorbance spectrophotometry; RNA integrity was checked on 1.2 % agarose gel electrophoresis. First-strand cDNA was synthesized from each total RNA (1 µg) using the QuantiTect reverse transcription kit (Qiagen), which allows the removal of genomic DNA contamination. Real Time PCR reactions were carried out in an Applied Biosystems 7500 Real Time System by using the Power SYBR® Green Master Mix PCR (Life Technologies). Each SYBR® Green reaction (20 µL total volume) contained 2 µL of 1:1 diluted cDNA as template, 12 µL of real-time PCR Master Mix, 1 µL of each of the forward and reverse primer (10 µM), and 4 µL of nuclease free water. Nuclease-free water for the replacement of cDNA template was used as a negative control. The thermal protocol was as follows: 1 min at 95 °C, followed by 40 cycles (15 s at 95 °C and 40 s at 60 °C). A melting curve analysis of PCR products was performed from 60 to 95 °C in order to ensure gene-specific amplification. For internal standard control, the expression of β-actin gene was quantified and used to normalize gene expression levels in each sample. Changes in the gene expression relative to the different samples were calculated according to the standard $2^{-\Delta\Delta C_t}$ method described by Schmittgen and Livak (2008). MT primers were designed on the N-terminal (forward primer) and C-terminal (reverse primer) regions of the *P. sicula* MT cDNA (Riggio et al. 2003); the sequences were: forward, 5′-ATGGATCCTCAGGACTGC-3′; reverse, 5′-ATCTG TATGAAAGAGCAATTAC-3′. ß-actin primers, designed on a homologous cDNA fragment (accession n. DQ0159 17), were: forward, 5′-TATCCACGAGACCACCTTC-3′; reverse, 5′-TCCACCAATCCAGACAGAG-3′.

Data are presented as mean ± standard error of the mean (SEM) from three separate experiments in each sample (n = 4). Statistical analyses were carried out by StatView software (Altura Software, Inc.), as described below.

Statistical analysis

Data analysis was performed by the independent samples *t* test, using the program contained in the package StatView (Altura Software, Inc.) at a significance level of $P < 0.05$. The two organic farming sites were analysed as independent cases. Metal concentrations and amount of MT transcripts were compared between each organic farming site and the control site using *t* tests.

Results

Localization of VTG and ERα mRNAs and proteins in liver

In situ hybridization experiments performed on liver sections from *P. sicula* males captured in the two organic farms showed the presence of transcripts for VTG (Fig. 1a, c) and ERα (Fig. 2a, c) in hepatocytes cytoplasm of both BIO-A and BIO-B animals. Such cells after immunolocalization experiments also showed a positive signal at cytoplasmatic level for both proteins (Figs. 1d, f; 2d, f). Sections of livers from control males remained completely unlabeled, both after ISH (Figs. 1b, 2b) and ICC (Figs. 1e, 2e) analyses. No signals were detected on sections incubated with sense riboprobes, as well as on sections for ICC obtained by omitting the primary antibodies. No changes in ISH and ICC signals were detected in DNase-treated sections (data not shown).

Cadmium and lead concentration in liver

Cd and Pb contents were determined in liver of every animal collected at the different sampling sites. Results are summarized in Fig. 3 with data given as group

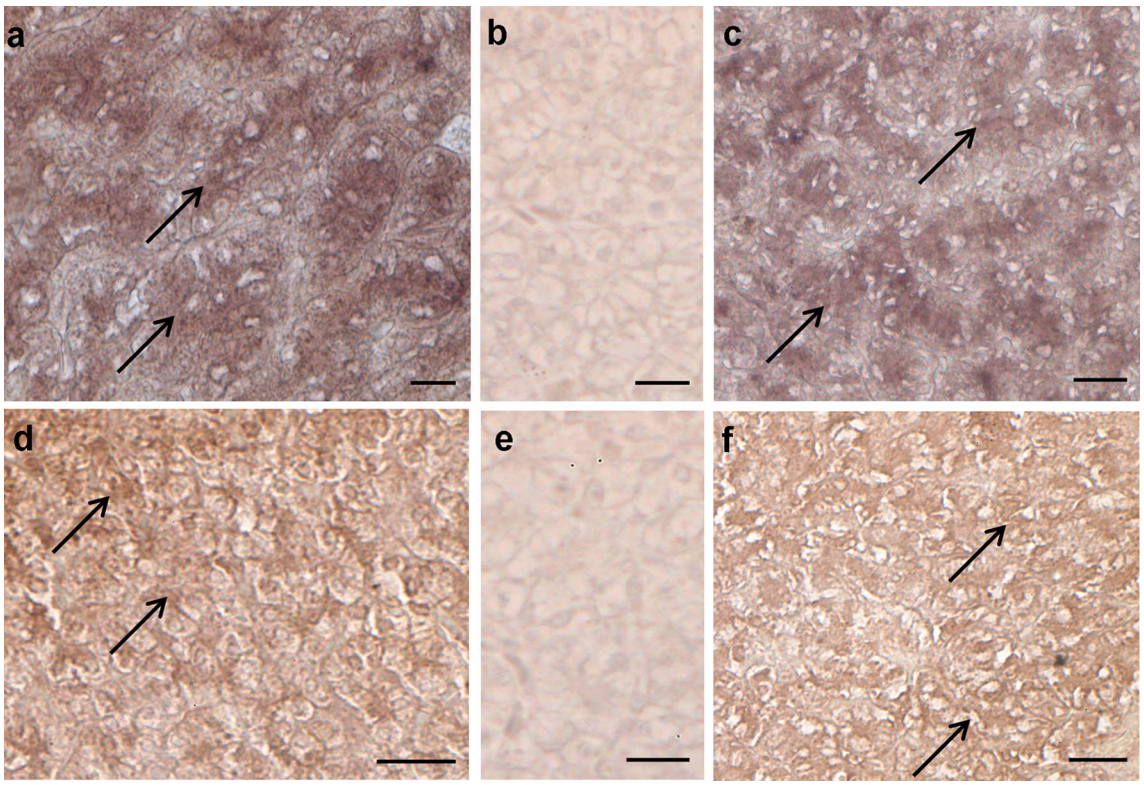

Fig. 1 Localization of VTG mRNA (**a**, **b**, **c**) and protein (**d**, **e**, **f**) in liver sections of *P. sicula* males. **a**, **d** BIO-A group; **c**, **f** BIO-B group; **b**, **e** *Ctr* control group. Both positive hybridization and immunolocalization signals, obtained as described under "Materials and methods" section, appear as *brown areas* (*arrows*). No signal was evident in the control animals. *Bars* 30 µm (Color figure online)

mean ± SD, n = 15. Both Cd and Pb levels were significantly higher in Ctr group with respect to BIO-A and BIO-B groups, whereas no differences in metals values were found between BIO-A and BIO-B groups (Table 1). In all instances Pb levels were lower than Cd levels.

Metallothionein expression in liver

To assess differences among sites in MT expression, a Real-Time PCR analysis was performed on transcripts from livers of the three different groups of lizards. Results demonstrated that in livers of animals captured in organic farms MT mRNA was less than half of the amount determined in livers of Ctr group animals, while no statistical differences were found between BIO-A and BIO-B groups (Table 2).

Localization of MT mRNA and protein in liver

Both in situ hybridization (Fig. 4a, b, d) and immunolocalization (Fig. 4e, f, h) experiments showed positive signals in the large Kupffer cells; some hepatocytes were also stained. No differences were observed in localization of MT mRNA and protein in the different groups of animals (Fig. 4). No signals were detected on sections incubated with sense riboprobes (Fig. 4c), as well as on sections for ICC obtained by omitting the primary antibody (Fig. 4g).

Discussion

Data herein described highlight that *P. sicula* males inhabiting organic farm areas show the typical hepatic biosynthetic alterations indicative of an estrogenic contamination. Hepatocytes, in fact, contain detectable amount of both VTG and ERα transcripts and proteins. On the other hand, the livers of the same animals show a very low level of the heavy metals Cd and Pb. In terrestrial vertebrates, Cd and Pb accumulation in liver is typical of organisms living in soils contaminated by anthropogenic activities such as smelting of ores, burning of fossil fuels, waste incineration; in addition, Cd reaches the soil through pesticides and herbicides (Frost and Ketchum 2000), Pb through the urban traffic (Pena-Fernandez et al. 2015). The amount of MT transcripts in these animals is significantly lower than that determined in lizards inhabiting rural sites in the outskirts of Naples. The soils of the two organic farms show a very low content of heavy metals (for BIO-A,

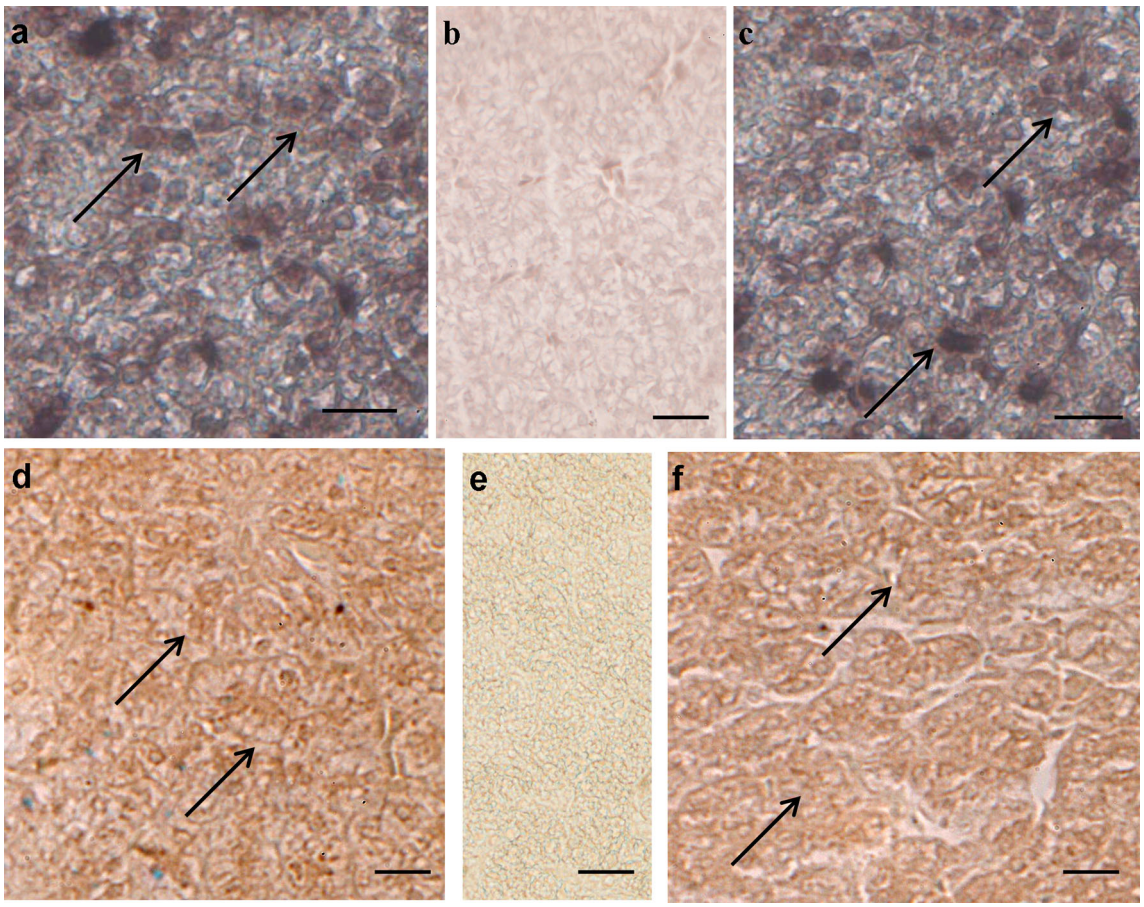

Fig. 2 Localization of ERα mRNA (**a**, **b**, **c**) and protein (**d**, **e**, **f**) in liver sections of *P. sicula* males. **a**, **d** BIO-A group; **c**, **f** BIO-B group; **b**, **e** *Ctr* control group. Both positive hybridization and immunolocalization signals, obtained as described under "Materials and methods" section, appear as *brown areas* (*arrows*). No signal was evident in the control animals. *Bars* 30 µm (Color figure online)

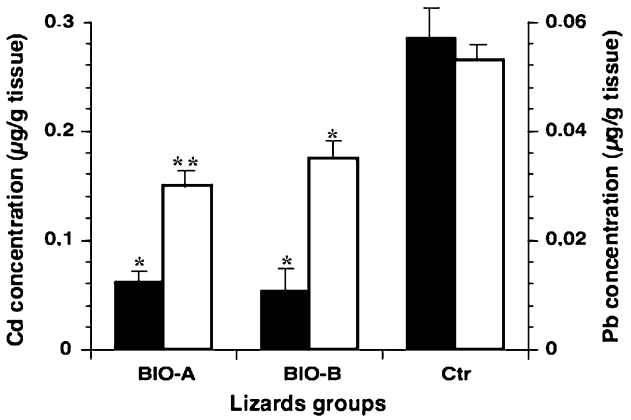

Fig. 3 Cadmium (Cd, *filled square*), and lead (Pb, *open square*), contents in *P. sicula* male livers. Values are expressed as mean ± SD (n = 15). *$P < 0.05$ versus Ctr group; **$P < 0.001$ versus Ctr group (see Table 1 for details on statistical analysis)

Cd was below the detection limit and Pb was 53.6 mg/kg soil; for BIO-B, Cd = 0.41 mg/kg and Pb = 20.3 mg/kg) and phenols (BIO-A: <0.010 mg/kg; BIO-B: <0.010 mg/kg), a condition typical of soils not contaminated with chemical fertilizers. Hence, the presence of substances able to induce the expression of the known biomarkers of estrogenic pollution i.e. ERα and VTG in the liver of lizard males, is most likely due to the massive use of manure as fertilizer. It has been suggested that VTG expression in oviparous males may be interpreted as a warning of reproductive adverse consequences (Cheek et al. 2001), and that over-activation of estrogen signaling through its receptors can have detrimental effects on the fertility parameters of male rats (Dumasia et al. 2015). Xenoestrogenic substances are responsible of feminization in fish (Hamilton et al. 2014) and are able to impair spermatogenesis in terrestrial vertebrates (Verderame and Limatola 2015). In *P. sicula*, an induction of apoptosis in male germ cells is also observed (Verderame and Limatola 2015) hence a prolonged use of manure could lead to changes in the spermatogenic cycle of these animals, endangering their reproduction and survival. In addition, an increased amount of estrogen plasma levels could have adverse effects also in female specimens, in particular on oogenesis

Table 1 Statistical values obtained from the two samples *t* test analysis on cadmium and lead contents in lizard livers

Correlated samples	Degrees of Freedom	*T* value		*P* value	
		Cadmium	Lead	Cadmium	Lead
BIO-A versus Ctr	6	3.277	4.273	0.0169*	0.0053*
BIO-B versus Ctr	6	3.210	3.441	0.0184*	0.0138*
BIO-A versus BIO-B	6	−0.041	−0.264	0.9688	0.8005

* Significance at $P < 0.05$

Table 2 Statistical values obtained from the two samples *t* test analysis on metallothionein mRNA content in lizard livers

Correlated samples	MT mRNA fold change	Degrees of freedom	T value	*P* value
BIO-A versus Ctr	0.37 ± 0.05	6	−3.798	0.0090*
BIO-B versus Ctr	0.45 ± 0.06	6	−4.088	0.0064*
BIO-A versus BIO-B	1.1 ± 0.1	6	−0.110	0.916

* Significance at $P < 0.05$; relative fold change is presented as mean ± standard error of the mean (SEM)

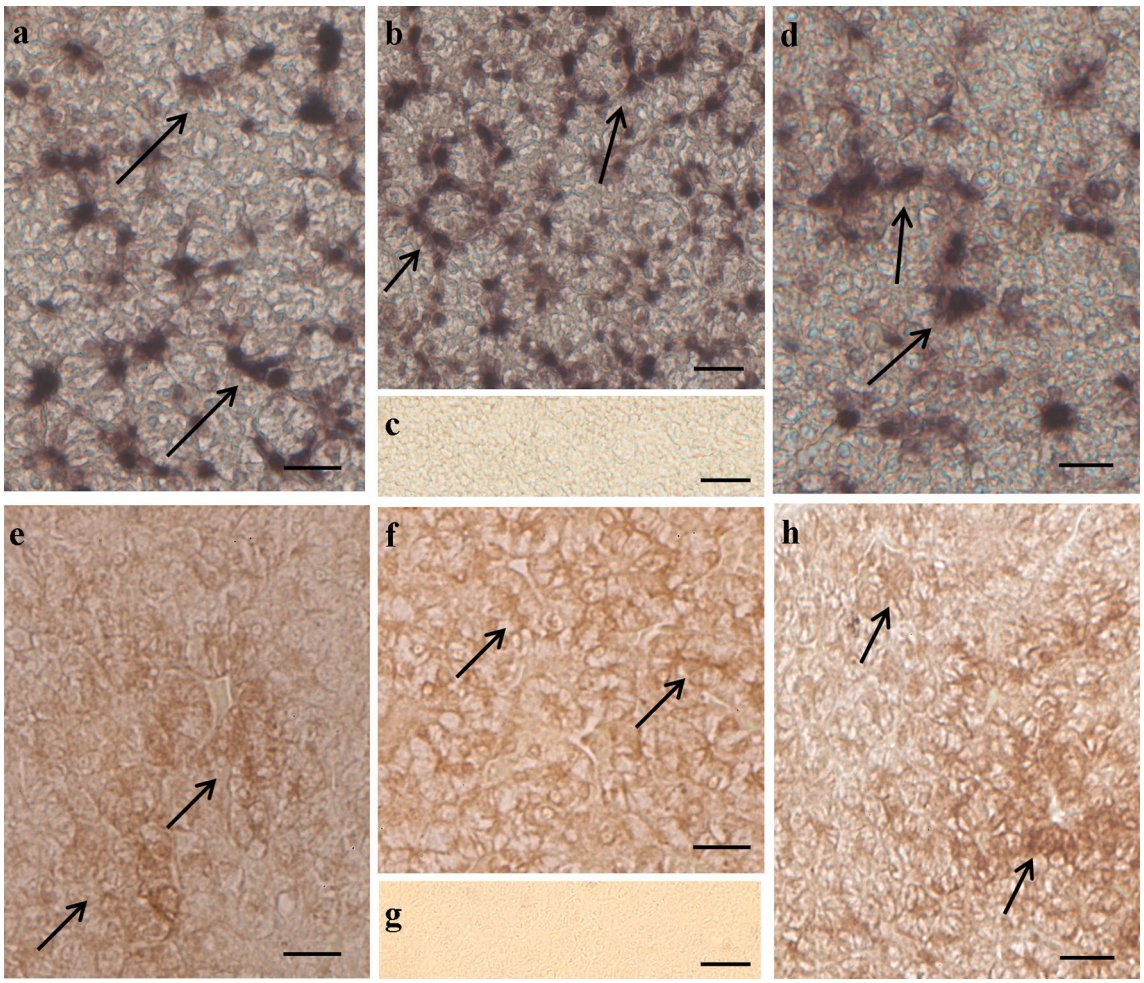

Fig. 4 Localization of MT mRNA (**a**, **b**, **d**) and protein (**e**, **f**, **h**) in liver sections of *P. sicula* males. **a**, **e** BIO-A group; **d**, **h** BIO-B group; **b**, **f** *Ctr* control group. Both positive hybridization and immunolocalization signals, obtained as described under "Materials and methods" section, appear as *brown areas* (*arrows*). Note the positive signals also in control animals. No hybridization and immunolocalization signals are evident in the control sections incubated with MT-sense riboprobe (**c**) and omitting anti-MT antibody (**g**), respectively. *Bars* 30 μm (Color figure online)

and oviposition. It has been demonstrated that exogenous estradiol increases shell thickness in laying hens (Wistedt et al. 2014). Moreover, yolk steroids of maternal origin may also influence sex determination and gonad differentiation (Ding et al. 2012; Navara 2013). On the whole, it can be hypothesized that exogenous estrogens could affect *P. sicula* embryonic development, offspring and reproductive cycle. In addition, it is not ruled out an increase of estrogens in animals bred in these organic farms (chicken, pig, sheep, cow) that could lead to reproductive failures for the same animals (Padmanabhan et al. 2010; Rosenfeld 2012) and to an undesirable higher content of estrogens in food chain, with possible risk for human consumers (Andersson and Skakkebaek 1999; Ibarreta et al. 2001; Aravindakshan et al. 2004).

In light of these data, it can be stated that organic farming avoids soil contamination by chemicals and heavy metals but, on the other hand, the use of manure as fertilizer may lead to an accumulation in soils of natural steroids and/or EDC that may affect reproductive processes. Organic farms should focus more on crop rotation and encouraging biological cycles and soil biological activity rather than using manure as fertilizer; it is also important to monitor the amount of estrogen derivatives in manure to avoid possible unwanted effects to wildlife and human health. In the recent years, it has been estimated that organic farming has grown by 8.9 % per year (Paull 2011); so, if nothing is done to mitigate the problem, soil contamination by steroids and EDC could soon represent a serious risk as currently is soil contamination by chemicals and heavy metals.

Acknowledgments Mariailaria Verderame was supported by the grant POR Campania FSE 2007/2013, TEMASAV (Tecnologie e Monitoraggio Ambientale per la Sostenibilità delle Aree Vaste) Project.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

Alvarez DA, Shappell NW, Billey LO, Bermudez DS, Wilson VS, Kolpin DW et al (2013) Bioassay of estrogenicity and chemical analyses of estrogens in streams across the United States associated with livestock operations. Water Res 47:3347–3363

Andersson AM, Skakkebaek NE (1999) Exposure to exogenous estrogens in food: possible impact on human development and health. Eur J Endocrinol 140:477–485

Aravindakshan J, Gregory M, Marcogliese DJ, Fournier M, Cyr DG (2004) Consumption of xenoestrogen-contaminated fish during lactation alters adult male reproductive function. Toxicol Sci 81:179–189

Asensio V, Rodriguez-Ruiz A, Garmendia L, Andre J, Kille P, Morgan AJ et al (2013) Towards an integrative soil health assessment strategy: a three tier (integrative biomarker response) approach with *Eisenia fetida* applied to soils subjected to chronic metal pollution. Sci Total Environ 442:344–365

Bargagli R, Brown DH, Nelli L (1995) Metal biomonitoring with mosses: procedures for correcting for soil contamination. Environ Pollut 89:169–175

Baronti C, Curini R, D'Ascenzo G, Di Corcia A, Gentili A, Samperi R (2000) Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environ Sci Technol 34:5059–5066

Bartelt-Hunt SL, Devivo S, Johnson L, Kranz WL, Mader TL, Shapiro CA et al (2013) Effect of composting on the fate of steroids in beef cattle manure. J Environ Qual 42:1159–1166

Brown V, Shurben D, Miller W, Crane M (1994) Cadmium toxicity to rainbow trout *Oncorhynchus mykiss* Walbaum and brown trout *Salmo trutta* L. over extended exposure periods. Ecotoxicol Environ Saf 29:38–46

Cardone A (2012) Testicular toxicity of methyl thiophanate in the Italian wall lizard (*Podarcis sicula*): morphological and molecular evaluation. Ecotoxicology 21:512–523

Caruso T, Migliorini M, Bucci C, Bargagli R (2009) Spatial patterns and autocorrelation in the response of microarthropods to soil pollutants: the example of oribatid mites in an abandoned mining and smelting area. Environ Pollut 157:2939–2948

Cheek AO, Brouwer TH, Carroll S, Manning S, McLachlan JA, Brouwer M (2001) Experimental evaluation of vitellogenin as a predictive biomarker for reproductive disruption. Environ Health Perspect 109:681–690

Combalbert S, Bellet V, Dabert P, Bernet N, Balaguer P, Hernandez-Raquet G (2012) Fate of steroid hormones and endocrine activities in swine manure disposal and treatment facilities. Water Res 46:895–906

Dallinger R, Berger B, Gruber C, Hunziker P, Sturzenbaum S (2000) Metallothioneins in terrestrial invertebrates: structural aspects, biological significance and implications for their use as biomarkers. Cell Mol Biol 46:331–346

Dallinger R, Lagg B, Egg M, Schipflinger R, Chabicovsky M (2004) Cd accumulation and Cd-metallothionein as a biomarker in *Cepaea hortensis* (Helicidae, Pulmonata) from laboratory exposure and metal-polluted habitats. Ecotoxicology 13:757–772

Darbre PD (2006) Metalloestrogens: an emerging class of inorganic xenoestrogens with potential to add to the oestrogenic burden of the human breast. J Appl Toxicol 26:191–197

Degen GH, Bolt HM (2000) Endocrine disruptors: update on xenoestrogens. Int Arch Occup Environ Health 73:433–441

Del Giudice G, Prisco M, Agnese M, Verderame M, Rosati L, Limatola E, Andreuccetti P (2012) Effects of nonylphenol on vitellogenin synthesis in adult males of the spotted ray *Torpedo* marmorata. J Fish Biol 80:2112–2121

Denslow ND, Chow MC, Kroll KJ, Green L (1999) Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. Ecotoxicology 8:385–398

Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M (1998) Identification of estrogenic chemicals in STW effluent: 1. Chemical fractionation and in vitro biological screening. Environ Sci Technol 32:1549–1558

Ding GH, Yang J, Wang J, Ji X (2012) Offspring sex in a TSD gecko correlates with an interaction between incubation temperature and yolk steroid hormones. Naturwissenschaften 99:999–1006

Dumasia K, Kumar A, Kadam L, Balasinor NH (2015) Effect of estrogen receptor-subtype-specific ligands on fertility in adult male rats. J Endocrinol 225:169–180

Frost HL, Ketchum LH (2000) Trace metal concentration in durum wheat from application of sewage sludge and commercial fertilizer. Adv Environ Res 4:347–355

Hamilton PB, Nicol E, De-Bastos ES, Williams RJ, Sumpter JP, Jobling S, Stevens JR, Tyler CR (2014) Populations of a cyprinid fish are self-sustaining despite widespread feminization of males. BMC Biol 12:1

Henle K, Klaver CJJ (1986) *Podarcis sicula* (RafinesqueSchmaltz, 1810) Ruineneidechse. Pp. 254–342 in Handbuch der Reptilien und Amphibian Europas. Böhme W Ed. Vol. 2/II. Echsen III (Podarcis), Aula, Wiesbaden pp 254-258

Henson MC, Chedrese PJ (2004) Endocrine disruption by cadmium, a common environmental toxicant with paradoxical effects on reproduction. Exp Biol Med 229:383–392

Hofer N, Diel P, Wittsiepe J, Wilhelm M, Kluxen FM, Degen GH (2010) Investigations on the estrogenic activity of the metallohormone cadmium in the rat intestine. Arch Toxicol 84:541–552

Iavicoli I, Fontana L, Bergamaschi A (2009) The effects of metals as endocrine disruptors. J Toxicol Environ Health B Crit Rev 12:206–223

Ibarreta D, Daxenberger A, Meyer HH (2001) Possible health impact of phytoestrogens and xenoestrogens in food. APMIS 109:161–184

Irwin LK, Gray S (2001) Vitellogenin induction in painted turtle, *Chrysemys picta*, as a biomarker of exposure to environmental levels of estradiol. Aquat Toxicol 55:49–60

Jobling S, Sumpter JP, Sheahan D, Osborne JA, Matthiessen P (1996) Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. Environ Toxicol Chem 15:194–202

Johnson MD, Kenney N, Stoica A, Hilakivi-Clarke L, Singh B, Chepko G et al (2003) Cadmium mimics the in vivo effects of estrogen in the uterus and mammary gland. Nat Med 9:1081–1084

Kammenga JE, Dallinger R, Donker MH, Kohler HR, Simonsen V, Triebskorn R, Weeks JM (2000) Biomarkers in terrestrial invertebrates for ecotoxicological soil risk assessment. Rev Environ Contam Toxicol 164:93–147

Li YX, Xiong X, Lin CY, Zhang FS, Wei L, Wei H (2010) Cadmium in animal production and its potential hazard on Beijing and Fuxin farmlands. J Hazard Mater 177:475–480

Matozzo V, Gagné F, Marin MG, Ricciardi F, Blaise C (2008) Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: a review. Environ Int 34:531–545

Menzi H, Haldemann C, Kessler J (1993) Schwermetalle in den Hofdüngern-ein Thema mit Wissenslücken. Schweizerische landwirtschaftliche Forschung 32:159–167

N'guessan YM, Probst JL, Bur T, Probst A (2009) Trace elements in stream bed sediments from agricultural catchments (Gascogne region, S-W France): where do they come from? Sci Total Environ 407:2939–2952

Navara KJ (2013) Hormone-mediated adjustment of sex ratio in vertebrates. Integr Comp Biol 53:877–887

Padmanabhan V, Sarma HN, Savabieasfahani M, Steckler TL, Veiga-Lopez A (2010) Developmental reprogramming of reproductive and metabolic dysfunction in sheep: native steroids vs. environmental steroid receptor modulators. Int J Androl 33:394–404

Paull J (2011) The uptake of organic agriculture: a decade of worldwide development. J Soc Dev Sci 2:111–120

Pena-Fernandez A, Lobo-Bedmar MC, Gonzalez-Munoz MJ (2015) Annual and seasonal variability of metals and metalloids in urban and industrial soils in Alcalà de Henares (Spain). Environ Res 136:40–46

Riggio M, Trinchella F, Parisi E, Filosa S, Scudiero R (2003) Accumulation of zinc, copper and metallothionein mRNA in lizard ovary proceeds without a concomitant increase in metallothionein content. Mol Repr Dev 66:347–382

Rosenfeld CS (2012) Effects of maternal diet and exposure to bisphenol A on sexually dimorphic responses in conceptuses and offspring. Reprod Domest Anim 4:23–30

Schiffer B, Daxenberger A, Meyer K, Meyer HH (2001) The fate of trenbolone acetate and melengestrol acetate after application as growth promoters in cattle: environmental studies. Environ Health Perspect 109:1145–1151

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101–1108

Simoniello P, Filosa S, Riggio M, Scudiero R, Tammaro S, Trinchella F, Motta CM (2010) Responses to cadmium intoxication in the liver of the wall lizard *Podarcis sicula*. Comp Biochem Physiol C 151:194–203

Simoniello P, Motta CM, Scudiero R, Trinchella F, Filosa S (2011) Cadmium-induced teratogenicity in lizard embryos: correlation with metallothionein gene expression. Comp Biochem Physiol C 153(1):119–127

Simoniello P, Filosa S, Scudiero R, Trinchella F, Motta CM (2013) Cadmium impairment of reproduction in the female wall lizard *Podarcis sicula*. Environ Toxicol 28:553–562

Simoniello P, Trinchella F, Filosa S, Scudiero R, Magnani D, Theil T, Motta CM (2014) Cadmium contaminated soil affects retinogenesis in lizard embryos. J Exp Zool A Ecol Genet Physiol 321:207–219

Tata JR, Smith DF (1979) Vitellogenesis: a versatile model for hormonal regulation of gene expression. Recent Prog Horm Res 35:47–95

Ternes TA, Kreckel P, Mueller J (1999) Behaviour and occurrence of estrogens in municipal sewage treatment plants-II. Aerobic batch experiments with activated sludge. Sci Total Environ 225:91–99

Trinchella F, Riggio M, Filosa S, Volpe MG, Parisi E, Scudiero R (2006) Cadmium distribution and metallothionein expression in lizard tissues following acute and chronic cadmium intoxication. Comp Biochem Physiol C 144:272–278

Valdehita A, Quesada-García A, Delgado MM, Martín JV, García-González MC, Fernández-Cruz ML, Navas JM (2014) In vitro assessment of thyroidal and estrogenic activities in poultry and broiler manure. Sci Total Environ 472:630–641

Verderame M (2014) The involvement of the androgen receptor in the secretion of the epididymal corpus in the lizard *Podarcis sicula*. Int J Zool. doi:10.1155/2014/457830

Verderame M, Limatola E (2010) Molecular identification of estrogen receptors (ERα and ERβ) and their differential expression during VTG synthesis in the liver of lizard *Podarcis sicula*. Gen Comp Endocrinol 168:231–238

Verderame M, Limatola E (2015) Interferences of an environmental pollutant with estrogen-like action in the male reproductive system of the terrestrial vertebrate *Podarcis sicula*. Gen Comp Endocrinol 213:9–15

Verderame M, Prisco M, Andreuccetti P, Aniello F, Limatola E (2011) Experimentally nonylphenol-polluted diet induces the expression of silent genes VTG and ERα in the liver of male lizard *Podarcis sicula*. Environ Pollut 159:1101–1107

Verderame M, Cafiero G, Limatola E (2012) Vitellogenin, oestradiol 17-β and its receptors in the lizard *Podarcis sicula*: cross-talk between growing oocytes and liver. In: Advances in Zoology Research Vol. 2, NOVA Publisher pp. 245-258

Verderame M, Angelini F, Limatola E (2014) Spermatogenic waves and expression of AR and ERs in germ cells of *Podarcis sicula*. Int J Zool. doi:10.1155/2014/965617

Vetillard A, Bailhache T (2005) Cadmium: an endocrine disrupter that affects gene expression in the liver and brain of juvenile *Rainbow trout*. Biol Reprod 72:119–126

Wallace RA (1985) Vitellogenesis and oocyte growth in nonmammalian vertebrates. Dev Biol 1:127–177

Weeks JM, Spurgeon DJ, Svendsen C, Hankard PK, Kammenga JE, Dallinger R, Kohler HR (2004) Critical analysis of soil invertebrate biomarkers: a field case study in Avonmouth, UK. Ecotoxicology 3:817–822

Wistedt A, Ridderstrale Y, Wall H, Holm L (2014) Exogenous estradiol improves shell strength in laying hens at the end of the laying period. Acta Vet Scand 56:34

Ying GG, Williams B, Kookana R (2002) Environmental fate of alkylphenols and alkylphenol ethoxylates: a review. Environ Int 28:215–226

Zhou S, Liu J, Xu M, Lv J, Sun N (2015) Accumulation, availability, and uptake of heavy metals in a red soil after 22-year fertilization and cropping. Environ Sci Pollut Res Int