

## Research Article

# Manure as Nonylphenol and Estradiol Promotes the Extrahepatic Synthesis of Vitellogenin in the Testis of a Terrestrial Vertebrate, the Lizard *Podarcis sicula*

Verderame M\*

Department of Biology, University Federico II - Via Mezzocannone 8, Italy

\*Corresponding author: Verderame M, Department of Biology, University Federico II - Via Mezzocannone 8, 80134- Napoli, Italy

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**Abstract**

In manure, the endocrine disruptors may have a dual source: as natural metabolites of the steroidal hormones present in the manure deposited by farm animals or of synthetic origin as the alkylphenols used for a variety of applications and consumer products. In particular, the EDC with estrogen-like activity cause a number of issues mainly related to the male fertility. This study aims to ascertain if an estrogenic environment is able to induce male gonad of the lizard *Podarcis sicula* to the synthesize Vitellogenin (VTG), the main biomarker of estrogenic exposure. From our investigations of *in situ* hybridization and immunohistochemistry emerges that VTG gene is transcribed and translated in the testis of lizards collected in organic farming where the manure is the unique fertilizer used and in the samples fed with food experimentally polluted by an estrogen-like substance i.e. nonylphenol. The same findings are recorded after the injection of estradiol-17 $\beta$ .

**Keywords:** Manure; Nonylphenol; Endocrine disruptor chemicals; Testis; Vitellogenin; Lizard

**Abbreviations**

E2: Estradiol 17- $\beta$ ; NP: Nonylphenol; EDC: Endocrine Disruptor Chemical; VTG: Vitellogenin; ISH: *In Situ* Hybridization; IHC: Immunohistochemistry

**Introduction**

Many substances in the environment mimic the endogenous hormones and interfere with the endocrine system [1] in particular with the males reproductive physiology [2,3]. These substances also known as Endocrine Disruptor Chemicals (EDC), originate from a variety of sources. They may be present in the co-formulants of pesticide as the alkylphenol polyethoxylates or, in form of metabolites of steroid hormones in the manure used for fertilization of the soils dedicated to organic farming [1,4,5].

The most commercially important alkylphenol is the Nonylphenol (NP), persistent and bioaccumulable, used primarily to produce surfactants for a wide variety of applications and consumer products [1]. Due to its structural similarity with estradiol-17 $\beta$ , the NP is able to bind to the estrogen receptors and to stimulate the transcription of the downstream genes [6] as the estrogen receptors itself and Vitellogenin (VTG).

Little attention is given to the metabolites of steroid hormones in manure. Farm animals excrete conjugated steroid hormones that persist in the manure for several months [7]. The main problem is that the conjugated and biologically inactive forms of the hormones are easily converted into free steroids by soil microorganisms as *Escherichia coli* [8-10].

Many biomarkers and bioindicator species have been identified to use in screening programs to determine the presence of the EDC

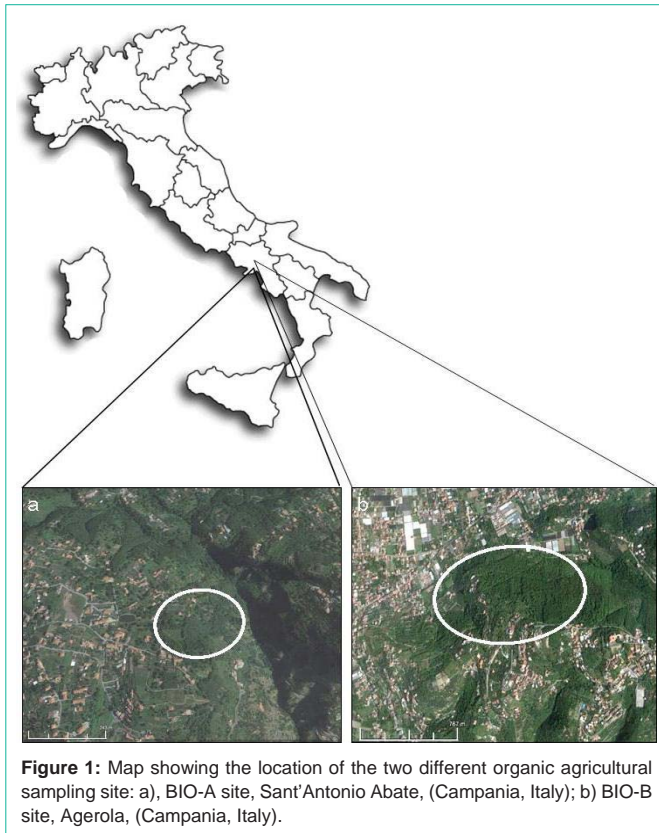
in the environment. The most validated biomarker of estrogenic exposure is the induction in males of Vitellogenin (VTG) the major egg yolk precursor in oviparous and ovoviviparous females [11,12]. Generally, VTG gene is expressed in the liver of mature females and is silent in males where it may be activated by estrogenic exposure at non physiological level. Most of the research in this field are conducted on aquatic or semiaquatic organisms and are focused on the hepatic induction of VTG in males living in polluted environments [13-15]. In the terrestrial experimental model such as the lizard *Podarcis sicula*, the E2 administration elicits (determines) the hepatic expression of VTG and ER $\alpha$  [24].

To date, the possible extrahepatic expression of VTG has received little or no attention [16]. In particular VTG was found in the testis of *Torpedo marmorata*, *Melanotaenia fluviatilis* and *Tanichthys albonube* [17-19] exposed to estrogenic chemicals. Our recent observations suggested that the treatment with estradiol-17 $\beta$  elicits the transcription and translation of VTG in the testis of *Podarcis* too [20].

So, the aim of the present research was to further investigate the possible induction of VTG in the lizard testis in two different experimental conditions. For this purpose *In Situ* Hybridization (ISH) and Immunohistochemistry (IHC) were used to detect the presence of VTG mRNA and protein in *Podarcis* samples collected in organic farming where the manure is the only fertilizer used or in samples fed with food polluted by NP. The results were compared with those obtained in the lizards injected with estradiol-17 $\beta$ .

**Materials and Methods****Animals and experimental treatment**

Sexually mature males *Podarcis sicula* (7-8 cm snout-vent length)



**Figure 1:** Map showing the location of the two different organic agricultural sampling site: a), BIO-A site, Sant'Antonio Abate, (Campania, Italy); b) BIO-B site, Agerola, (Campania, Italy).

were caught during their mating period (May-June) in two organic farms both located in Sorrento Peninsula (Campania, Italy): 10 animals were from a site near Gragnano (BIO-A) and 10 from a site near Agerola (BIO-B group) (Figure 1). The two sites are certified as exclusively organic farms by the Italian Department of Agriculture. Both farms are large (approx. 800 m<sup>2</sup>), perched on a hill, far away and isolated from non-organic crops. These sites use the manure of animals bred in the same farms as fertilizer.

Further, thirty lizards were caught on the outskirts of Naples in an uncultivated with no previous signs of estrogenic contamination [21-23]. These wild caught animals were randomly divided into three groups. First group of ten lizards undergoing treatment with NP (Etravon-Syngenta, Italy), was fed every other day for 2 weeks with larvae of *Tenebrio molitor* sprayed with an aqueous NP solution (0.25%); a drinking trough containing water polluted with NP (0.05%) was always available. The live mealworms were sprayed with NP outside the terraria and then transferred inside. Their ingestion by the lizards, which eat only living and mobile larvae, was observed. Second group of ten samples were experimentally treated intraperitoneally with 17 $\beta$ -estradiol (168 ng/100 ml) in reptile physiological solution (NaCl 0.07%) every other day for 2 weeks [24] as control of the estrogen response. Third group of ten untreated wildlife animals, were considered as a control of the reproductive stage.

All the animals were killed by decapitation after anesthesia in ice and the testes were immediately excised and processed for ISH and IHC investigations. We were authorized to capture the animals for experimental treatments by the Italian Ministry of the Environment (auth. SCN/2D/2000/9213) 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).

### In situ hybridization

The testis were fixed for about 6 h in Bouin's solution and then dehydrated in graded ethanol and embedded in paraffin wax. ISH was performed on adjacent sections with VTG cDNA probe, as previously described [21,23,25]. Briefly, the sections dewaxed in Xylene solution and rehydrated in graded ethanol, were treated with proteinase K (10 lg/ml) at 50°C for 10 min. Digoxigenin (DIG)-labeled probes were used at a concentration of 80 ng/100 $\mu$ l 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 1x; tRNA 100  $\mu$ g/ml; ss-DNA 100  $\mu$ g/ml) overnight at 50°C in a moist chamber. The slides were incubated with RNase mix at 37°C for 30 min and in the same mix without RNase at 37°C for 30 min, washed in 2x SSC for 3 min, in 0.1x SSC at 60°C 15 min, and in NTP (Tris-HCl 0.1 M, pH 7.5; NaCl 0.15 M) and then incubated in 2% blocking solution (Roche Diagnostics, Mannheim, Germany) in maleic acid buffer (0.1 M maleic acid; 0.15 M NaCl, pH 7.5) for 1 h. The sections were kept overnight at 4°C with an alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche Diagnostics) (1:2500) in blocking solution and rinsed in NTP buffer for 30 min and in NTM buffer (Tris-HCl 100 mM, pH 9.5; MgCl 50 mM; NaCl 100 mM) for 30 min. Finally, the sections were kept in the color detection substrate solution BCIP/NBT (nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate) in the dark at room temperature as recommended by the manufacturer (Roche) in NTM until appearance of the color. For negative control, the hybridization solution did not contain cDNA probes.

### Immunohistochemistry

For VTG localization, testis sections were incubated with an homologous primary anti-VTG antibody (1:1000) [26] 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). To detect antigen, ultrasensitive ABC staining reagent kit (Pierce, USA) and diaminobenzidine (DAB 1 mg/ml; Sigma) was used. Negative control sections were obtained by omitting incubation with primary antibody.

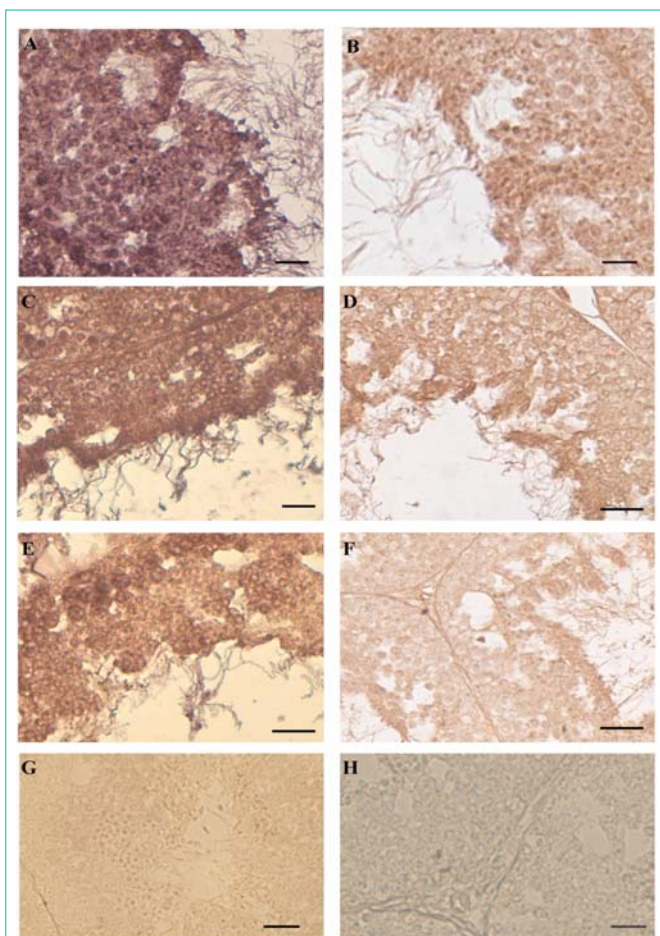
### Results

In all the samples captured in BIO areas (Figure 2A,B) or exposed to the NP-polluted diet (Figure 2C,D) VTG-mRNA (Figure 2A,C) or VTG protein (Figure 2B,D) were detected in all cells of the seminiferous epithelium. In the animals injected with E2, showing a reduction of the seminiferous epithelium as the NP samples, the same positivity to ISH (Figure 2E) and IHC (Figure 2F) was recorded.

In the testis of untreated wild males no VTG-mRNA neither VTG protein was detected (one for all is depicted in Figure 2G). The sections incubated without VTG probe or without primary antibody as a control of reactions displayed always negative results (one for all is depicted in Figure 2H).

### Discussion

The present results evidencing the expression of VTG in the testis



**Figure 2:** *In situ* hybridization and immunocytochemistry in the testis of *Podarcis*. To the left of the panel *in situ* hybridization with VTG probe: in BIO (A), NP-treated (C) and E2-treated (E) samples all germ cells of the seminiferous epithelium express VTG-mRNA. No VTG-mRNA is detected in wildlife untreated males (G). To the right of the panel immunohistochemistry with anti-VTG antibody: in BIO (B), NP-treated (D) and E2-treated (F) all germ cells synthesize VTG-protein. No positivity was evident in the control reactions (one for all is depicted in H). The bar is 30  $\mu$ m.

of lizards living in the areas of organic farming where the manure is the unique fertilizer used, suggest that the manure may have estrogenic action as NP and E2.

Therefore, being the testis the site of germ cells production, this anomalous synthesis of VTG may threaten the fertility and then the continuity of the specie.

*Podarcis* mature females physiologically synthesize VTG during the breeding season. In this period each ovulatory wave is characterized by a peak of E2 and VTG, in non-breeding period the levels of endogenous E2 are low and plasma VTG is absent [27,28]. In wild males this protein is never detected in any tissue neither when the levels of circulating E2 are the highest for males during the post-mating refractory period [29]. Nevertheless it's widely demonstrated in the same lizard [24,25] as in other oviparous vertebrates [12,27,30-33] that the estrogen are able to trigger the expression of VTG gene in the liver of males.

In the referred experimental plain were considered two conditions in which the wild animals may live in the environment polluted by

estrogen-like substances. In the first case, the lizards were captured in the fields devoted to BIO-farming where the manure is the main used fertilizer. In the second, the potential conditions to which the terrestrial vertebrates may be exposed by eating and drinking in xeno-estrogenic polluted fields were experimentally mimicked. The results of ISH and IHC undoubtedly showed that, both in BIO animals and NP-exposed males, the testis was able to transcribe and translate VTG as in the E<sub>2</sub> treated males. This confirms once again the xeno-estrogenic action of the manure and NP already observed by us in the liver of this terrestrial vertebrate [25,34].

The researches on the extrahepatic synthesis of VTG are really scarce and are conducted mainly in male fishes exposed to E2 or NP [35,36].

In the samples of *Podarcis* here analyzed, the presence of VTG-mRNA in all stage of spermatogenesis proves clearly that the VTG synthesis takes place *in situ* and therefore the germ cells are the target of the estrogenic action of manure and NP.

Moreover, the anomalous presence of VTG in the testis may be prelude of reproductive impairment, compromising the fertility of *Podarcis*. It is known that in this lizard the treatment with E2 or NP induces a slowdown of spermatogenesis with consequent alteration of the testicular structure [20,37]. Also in the samples here examined it is evident that E2 or NP during the reproductive period determines a reduction of the seminiferous epithelium affecting the testicular structure making it similar to that of the non-reproductive period. Further in rat, NP induces impairment of sperm function and motility [38].

Lizards are important components of the food webs in most ecosystems. They fill a critical role both as predator and prey species: they are an important prey for birds, snakes and other animals, and are important predators of insects. *Podarcis* can be directly exposed through various routes, including: ingestion of contaminated food and/or soil, inhalation, dermal exposure. So, *Podarcis* appears to be very useful terrestrial non-mammalian bio-indicator specie and VTG, the rapid inducible biomarker, is once again a valuable assay to easily verify the damages caused by EDC.

In synthesis, taken together these results open a number of issues on the use, both in BIO-farming and in the traditional agriculture, of fertilizers which can cause alteration of the endocrine homeostasis. All this, besides to represent a serious problem for the food chain, poses a serious question for the continuity of this species as of the other animal species. Their disappearance in fact, could have an ecological importance greater than what one might think since the removal of any species from its ecosystem can drastically alter the populations of other organisms.

## Conclusion

In conclusion, these results demonstrated for the first in a terrestrial vertebrate the ability of the testis to synthesize VTG. In males, the activation of an estrogen-dependent gene i.e. VTG, in the two reported experimental condition, may be due to the presence of estrogen metabolites in manure used to fertilize the fields on which the lizards were collected for this study or to diet experimentally polluted by an estrogen-like substance as NP.

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