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Interferences of an environmental pollutant with estrogen-like action in the male reproductive system of the terrestrial vertebrate *Podarcis sicula*

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ABSTRACT

Nonylphenol (NP) is classified among the endocrine disruptor chemicals with estrogen-like properties. It is widely used in many industries and to dilute pesticides in agriculture, and is known to affect the reproductive system of many aquatic and semi-aquatic organisms. This study aimed to verify how NP, administered via food and water, may interfere with the reproductive cycle of a terrestrial vertebrate. Our model was the male Italian wall lizard *Podarcis sicula*, a seasonal breeding species that may be naturally exposed to environmental pollution. From our findings it emerges that an NP-polluted diet administered during the mating period causes in this lizard a slowdown of spermatogenesis and affects the testicular and epididymal structure, making it similar to that of the non-reproductive period. The distribution in the testis and epididymis of mRNA for steroid hormone receptors, i.e., estrogen α and β and androgen receptors, was also investigated. NP treatment inhibits the expression of AR, ERα, and ERβ-mRNA in spermatogonia and primary spermatocytes and causes a switch-off of the secretory activity of the epididymal *corpus* by inducing the expression of ERα.

1. Introduction

Nonylphenol (NP) is a xenobiotic compound belonging to the class of alkylphenols which are persistent and non-biodegradable environmental contaminants. NP is classified among the endocrine disruptor chemicals (EDCs) as a xeno-estrogenic substance. Due to its molecular similarity with estradiol-17β (E_2), NP mimics E_2 action, also competing for the ligand binding site of the estrogen receptors (ERs) (White et al., 1994; Lee and Lee, 1996) even if its estrogenic potency, both *in vivo* and *in vitro* systems, is less than natural estrogens (~10,000 fold lower than estradiol) (Soto et al., 1995; Jobling et al., 1996; Blom et al., 1998; Sohoni and Sumpter, 1998; Laws et al., 2000; Arukwe et al., 2001; Thorpe et al., 2001).

NP is widely used in the cosmetic, textile and leather processing industries (Jobling and Sumpter, 1993; Ying et al., 2002; Vazquez-Duhalt et al., 2005), and in agriculture as a co-formulant of pesticides or emulsifier for teat dips; it accumulates in rivers, soil, groundwater, sediment, atmosphere, sewage sludge, drinking water (Duan et al., 2014; Jin et al., 2014; Liu et al., 2014; Zhang et al., 2014), and through the food chain in organisms (Guenther et al., 2002; Muncke, 2009). In the European Union the use of NP has been limited to 0.1% in open systems (EU: Directive 2003/53/EC of the European Parliament, 2003). From a recent report by Greenpeace (January 2014) it emerged that nonylphenol ethoxylates (NPEs; up to 17,000 mg/kg) were found in many children's clothing and footwear made also by the major known brands; the washing of these clothes releases NPEs into the public wastewater systems of the countries where these products are sold.

EDCs with estrogen-like action are known to affect the male reproductive system (Carlsen et al., 1992; Milnes et al., 2006; Tripathi et al., 2009; Lagos-Cabre and Moreno, 2012) but the mechanisms by which NP interferes is not fully understood (Hong-Xia et al., 2013). Furthermore, the finding in male plasma of vitellogenin (VTG), a typical female estrogen-dependent protein, is considered the main biomarker of environmental pollution by xeno-estrogenic substances (Christiansen et al., 1998; Kinnberg et al., 2000a; Del Giudice et al., 2011; Verderame et al., 2011).

Most of the research in this field has been conducted on aquatic vertebrates collected or experimentally exposed to polluted environment (Ahel et al., 1993; Jobling et al., 1996; Christiansen et al., 1998; Arukwe et al., 2000a,b; Kinnberg et al., 2000a,b; Seo et al., 2006; Ruggeri et al., 2008; El-Sayed Ali et al., 2014; Staniszewska et al., 2014) while few studies have been carried out on terrestrial vertebrates (Han et al., 2004; Razia et al., 2006; Iguchi et al., 2006; De Falco et al., 2014) for whom the soil and feeding are the main routes of exposure.

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The aim of the present research was to investigate the effects of NP-polluted diet (food and drink) on the morphology of testis and epididymis in the lizard *Podarcis sicula* that is widespread in Italy and feeds on insects, plants and pedofauna. This lizard is a seasonal breeding species, its mating period lasting from spring to early summer. In these months an intense spermatogenic activity takes place in the testis and the epididymis changes in relation to the passage of the spermatozoa (Angelini and Botte, 1992). Further, to clarify some aspects by which the NP could interfere with the reproductive cycle of this lizard we also ascertained the expression of androgen and estrogen receptor in these compartments.

2. Materials and methods

2.1. Animals and experimental protocol

Male *P. sicula* lizards (about 7 cm snout-vent) were captured near Naples (Campania, Italy) during the mating period (early May), randomly divided into three groups, kept in terraria and exposed to natural temperature and photoperiod. The experimental group ($n = 9$), 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. A control group (C1; $n = 5$) was fed with non-polluted food and received fresh water for 2 weeks; another untreated control group (C2; $n = 5$) was sacrificed at time 0, 2 days after capture.

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

2.2. Histology

The specimens were fixed in Bouin's fluid (Mazzi, 1977), alcohol dehydrated and paraffin embedded. Sections 7 mm in thickness were obtained with Reichert–Jung 2030 microtome. Some sections were stained with Mallory's trichrome modified by Galgano (Mazzi, 1977); others were processed by *in situ* hybridization (ISH) and TUNEL test. All the histological results were examined using a Nikon-MicroPhot-FXA. To evaluate the size of Sertoli cells the microscope was equipped with a micrometer ocular (Leica). Student's *t*-test was used to determine which values significantly differed from controls; one-way analysis of variance (ANOVA) was used (GraphPad Prism 5 software), and significance of tests was accepted at $p < 0.05$.

2.3. In situ hybridization (ISH)

ISH was performed on adjacent sections with homologous AR, ERα or ERβ probes (Verderame et al., 2011,2012; Verderame, 2014). Briefly, the dewaxed sections were treated with proteinase K (10 μg/ml) at 50 °C for 10 min. Digoxigenin (DIG)-labeled probes 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×; tRNA 100 μg/ml; ss-DNA 100 μg/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 2× SSC for 3 min, in 0.1× 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′-indolyphosphate) in the dark at RT as recommended by the manufacturer (Roche) in NTM until appearance of the color.

2.4. TUNEL test

The TUNEL test was performed with the TdT-FragELTM DNA Fragmentation Detection Kit as recommended by the manufacturer (Calbiochem, Germany). Briefly, the sections were rinsed with Tris-buffered saline (TBS; 20 mM Tris, pH 7.6; 140 mM NaCl) and treated with proteinase K (20 μg/ml) for 20 min. After a wash in Tris (10 mM, pH 8), the sections were incubated with 3% H_2O_2 for 4 min to inactivate the endogenous peroxidases. The slides were incubated for 5 min with TdT buffer (200 mM Na-cacodylate; 30 mM Tris; 0.3 mg/ml BSA, and 0.75 mM $CoCl_2$, pH 6.6) and then covered with 60 μl of TdT and biotinylated dUTP (3 μl TdT enzyme and 57 μl Labeling Reaction mix) and kept for 1.5 h at 37 °C in a moist chamber. Negative controls were obtained with biotinylated dUTP in TdT buffer without TdT enzyme. The reaction was stopped by incubating the slides with stop solution (0.5 M EDTA, pH 8.0) for 5 min at room temperature. The sections were blocked in 4% BSA and then incubated with conjugated streptavidin–horseradish peroxidase for 30 min. The reactions were finally revealed with 3,3-diaminobenzidine (DAB) solution and counterstained with methyl green.

3. Results

Both the untreated animals killed at time 0 (C2) and males that received a non-polluted diet for 2 weeks (C1) showed the same features. Hence from now on they will be indicated as control males.

3.1. Testis: Histology and mRNA distribution of AR and ERs

The testes of the untreated control males stained with Mallory's trichrome showed the seminiferous epithelium filled with all germ cells from spermatogonia (spg) to spermatozoa (spz) as expected in the mating period (Fig. 1a). In the animals exposed to the NP-polluted diet the lumen of the tubules was wide (Fig. 1b), the seminiferous epithelium was reduced in thickness and several empty spaces were evident (Fig. 1b), likely due to a decrease in the amount of the germ cells even if all stages of their differentiation were detectable. Among the germ cells many spermatids (spd) were positive to the TUNEL test (Fig. 1c) unlike the untreated control animals (Fig. 1d). Sertoli cells appeared hypertrophic and enlarged (Fig. 1e and f); statistical analysis performed by measuring the diameter of 30 Sertoli cells reveals a size of about 5.14 ± 0.53 μm in control samples and of 10.74 ± 0.54 μm in NP-treated males.

In the control males the expression of ERα (Fig. 2a), ERβ (Fig. 2b) and AR (Fig. 2c) in the seminiferous epithelium occurs in all germ cells from spg to spz. In the NP-treated samples the spg and primary spermatocytes (spcI) lacked the ERα, ERβ or AR mRNA transcripts whereas they were present in the secondary spermatocytes (spcII), spd and spz (Fig. 2d–f).

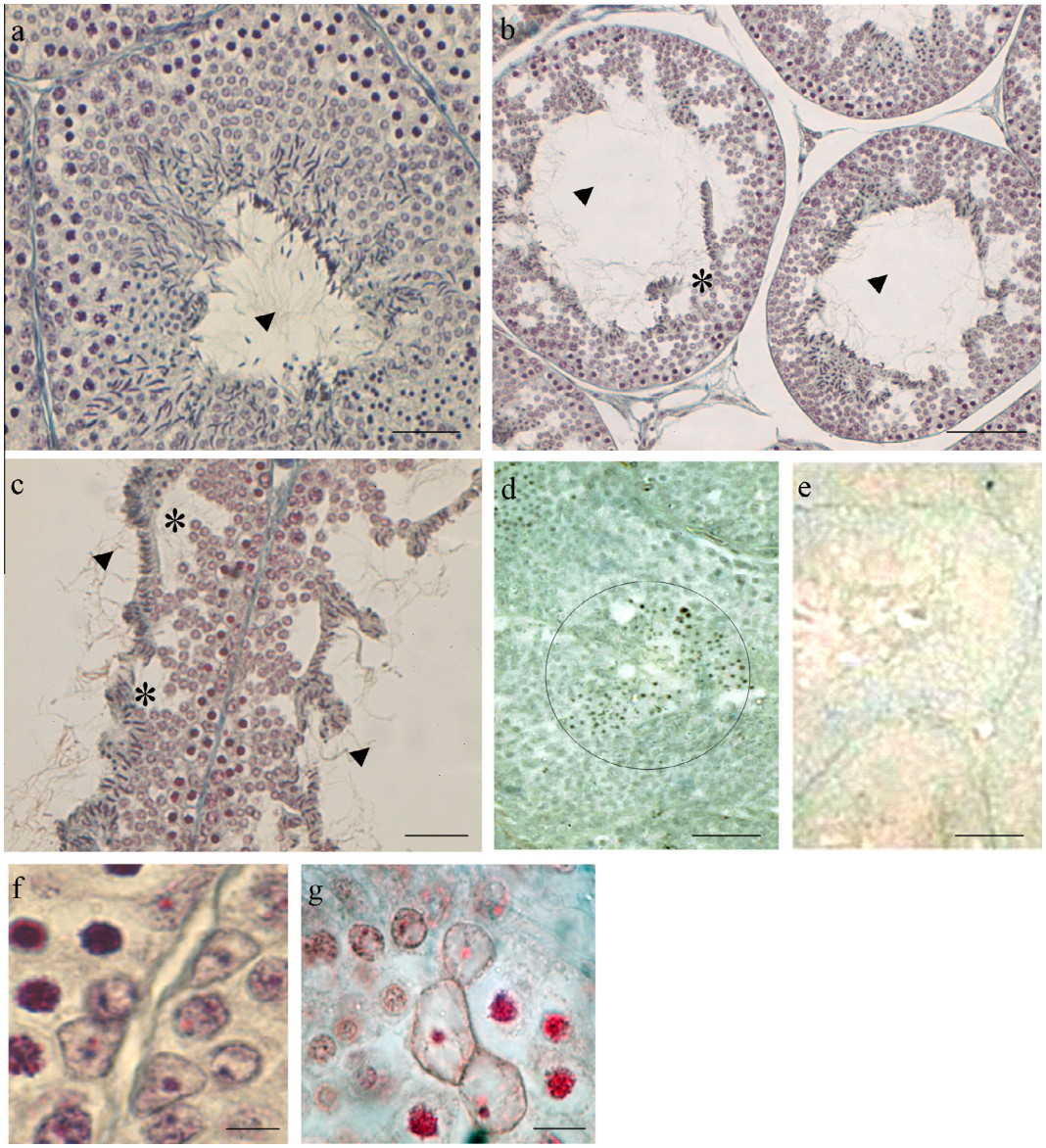

Fig. 1. Histology of the *Podarcis* testis. (a) Untreated control males, mating period: all stages of spermatogenesis are evident in the seminiferous epithelium; note the narrow lumen of the tubule (). (b) NP-treated samples: in the seminiferous epithelium several empty spaces (*) are evident; note the enlarged lumen of the tubule (). (c) As (c), high magnification. (d) TUNEL assay in NP-treated samples: note the positive spd (↑). (e) In the untreated control males no germ cells are positive to TUNEL assay. (f) Sertoli cells from untreated and (g) from NP-treated males. Mallory's trichrome staining (a–e); TUNEL assay (c and d). The bar is 30 μm for (a)–(c), (f) and (g) and 5 μm for (d) and (e).

3.2. Epididymis: Histology and mRNA distribution of AR and ERs

The epididymal channel of *Podarcis* is partitioned into *efferent ductules, corpus and cauda*. In control samples during the mating period the *corpus* shows the well-known spectacular variation with elongated lining cells, engaged in a massive secretory activity, and a lot of spermatozoa and secretory granules in the lumen (Fig. 3a). In this period the secreting cells lacked ERα expression (Fig. 3c) whereas AR and ERβ mRNAs were always present, thereby confirming our previous results (Verderame et al., 2012; Verderame, 2014).

In the lizards exposed to the NP-polluted diet during the mating period the epithelium of the epididymal *corpus* appeared cubic and non-secreting with no, or rare, sperms inside the lumen resembling the natural post-reproductive period (Fig. 3b); in these non-secreting cells ERα mRNA was detected (Fig. 3d) together with ERβ (Fig. 3e) or AR (Fig. 3f) transcripts. The epithelium of the *efferent ductules* and *cauda* showed no histological changes in respect to the controls and were positive to the three probes as expected.

4. Discussion

The present results highlight the fact that an experimental NP-polluted diet administered during the full mating season affects the morpho-physiology of the testis and epididymis of the lizard *P. sicula*. This lizard is an important component of the terrestrial ecosystem and an integral part of the food chain, preying on many invertebrates and being itself prey to vertebrates: all this makes *Podarcis* a good bio-indicator species. In our experimental plan we replicated the condition to which lizards could be naturally exposed by eating pedofauna and drinking water in NP-polluted environments. Our results are the first to analyze the effects of a xeno-estrogenic substance on the gonads of a terrestrial vertebrate.

Our choice of NP concentrations made allowances for the fact that, in agriculture, treatments may be repeated and that NP, which is persistent and accumulable, shows a half-life of over 60 years in sediment (Shang et al., 1999). Moreover, run-off from

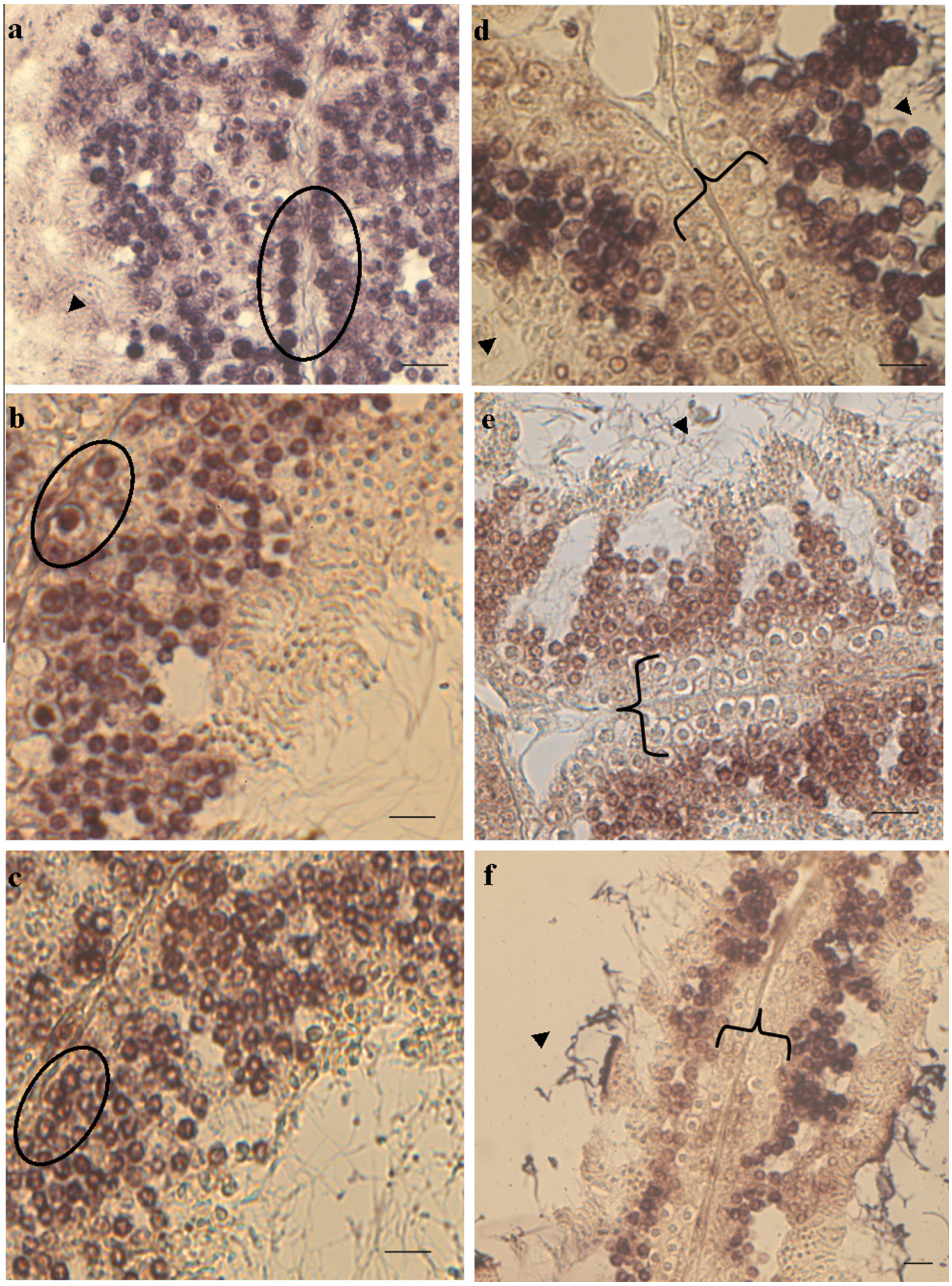

Fig. 2. *In situ* hybridization with ERα, ERβ or AR homologous probes. To the left of the panel: untreated control males: all germ cells in the seminiferous epithelium express ERα (a) ERβ (b) or AR (c) mRNA. In particular, note the intense positivity in spg (○) on the basis of the tubules. To the right of the panel: NP-treated males; ERα (d), ERβ (e) or AR (f) mRNA is unexpressed in spg and spcI on the basis of the tubules ({) and detected toward the lumen () in spcII, spd and spz. The bar is 30 μm.

fields treated with NP as co-formulant in pesticides causes its accumulation. Further, the preference of a more concentrated solution to spray the food was due to the fact that the live mealworms, sprayed with NP outside the terraria, when transferred inside move in the soil and remove some of the NP from their body before being eaten by the lizards. During the 2 weeks of treatment, the animals ate whenever food was made available (every other day) and always showed normal mobility in the terraria.

In wild samples of the *Podarcis* lizard, intense spermatogenic activity takes place in the testis during the mating period and a large amount of germ cells are always present in the seminiferous epithelium (Angelini and Botte, 1992). The reduction in thickness of the seminiferous epithelium and the presence of empty space among the germ cells in samples treated with an NP-polluted diet may indicate a failure in the replacement of cells from the basis of the tubules. A high degree of testis vacuolization and impairment of spermatogenesis were also reported in aquatic species exposed to natural or experimentally polluted environments or injected with NP (Bjerregaard et al., 2006; Christiansen et al., 1998; Kinnberg et al., 2000b).

Moreover, in *Podarcis*, despite the presence of all the stages of germ cell differentiation, the round spermatids are apoptotic (positive to TUNEL test) and ISH responsive even in the few tubules that show minor changes. In TUNEL-positive germ cells of rat the ERβ immunoreaction is enhanced, suggesting that ERβ is involved in the induction of germ cell apoptosis (Selva et al., 2004; Sasso-Cerri, 2009). In calvarial osteoblasts NP up-regulates Bax/Bcl2 and triggers apoptosis (Sabbieti et al., 2011). In some fish the increase in the amount of TUNEL-positive testicular germ cells was related to the NP-dependent gonadotoxicity (Weber et al., 2002; Kaptaner

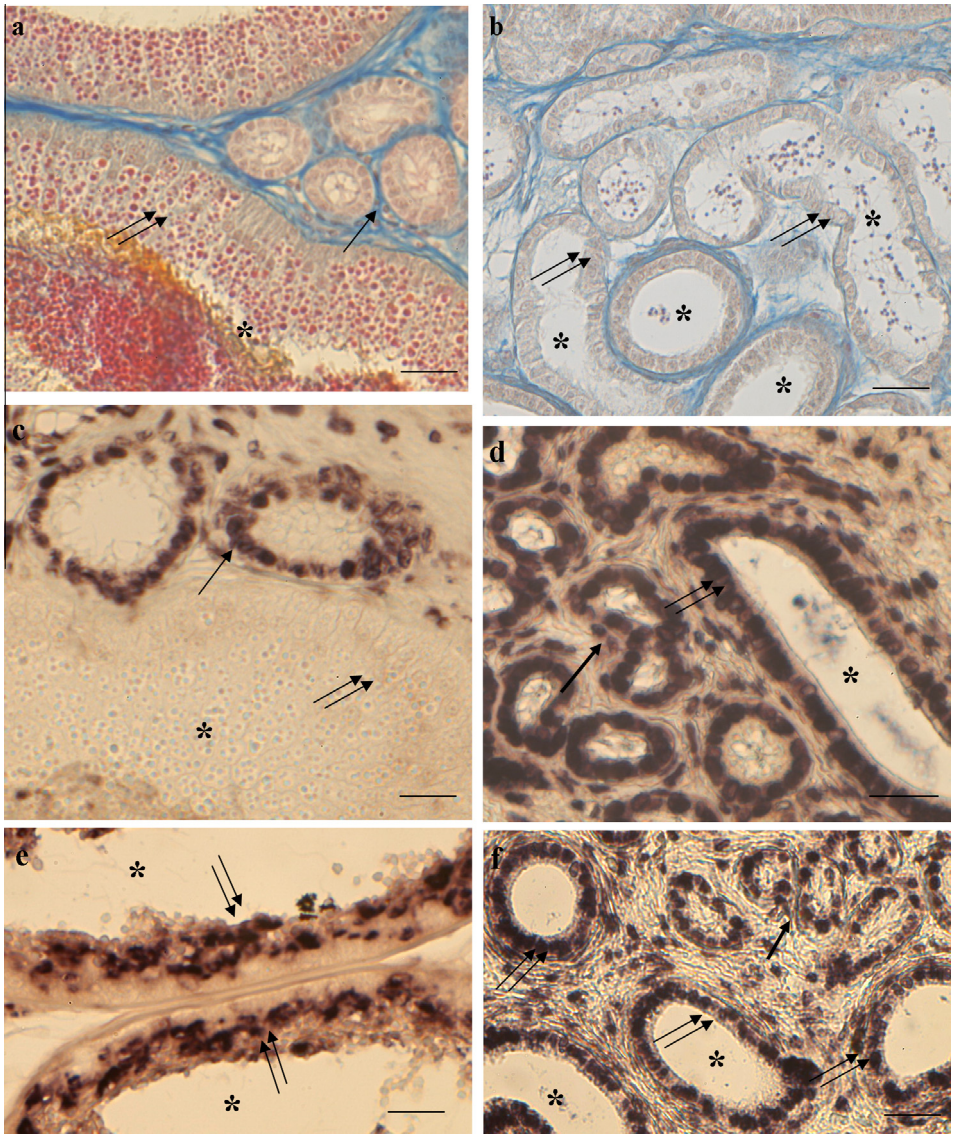

Fig. 3. *Podarcis* epididymis. (a) Untreated control males: the epithelium lining the *corpus* is cylindrical with elongated cells secreting a lot of granules (↑↑) in the lumen (*) full also of sperms. The *efferent ductules* show a cubic ciliate epithelium (↑). (b) NP-treated males: the epithelium of the *corpus* (↑↑) is regressed and few granules and sperm are present in the lumen (*). (c) Untreated control males: the ERα mRNA is unexpressed in the secreting cells of the *corpus* (↑↑) and expressed in the *efferent ductules* (↑). (d–f) NP-treated samples: (d) ERα expression takes place in non-secreting *corpus* (↑↑) and remains in the *efferent ductules* (↑), (e) ERβ mRNA in non-secreting *corpus* (↑↑), (f) AR mRNA in non-secreting *corpus* (↑↑) and in the *efferent ductules* (↑). Mallory's trichrome staining (a and b); *in situ* hybridization (c–f). The bar is 30 μm.

and Unal, 2011). The sensitivity of the testis to environmental pollutants, as of other endocrine glands of *Podarcis* (De Falco et al., 2007; Sciarrillo et al., 2008), is also shown by the induction of apoptosis in male germ cells by methyl thiophanate, a broad-spectrum fungicide used to prevent plant diseases (Cardone, 2012).

Furthermore, in samples subjected to the NP-polluted diet our microscopic observations followed by statistical analysis showed an increase in size of Sertoli cells. In teleosts, it is well established that NP causes hypertrophy of Sertoli cells related to the increase in their activity of phagocytosis (Russel et al., 1990; Christiansen et al., 1998; Kinnberg et al., 2000a,b; Miura et al., 2005; Bjerregaard et al., 2006).

In *Podarcis* the NP-polluted diet also affects the expression of the androgen and estrogen receptors in male germ cells. In the testis of untreated samples the distribution of ERα, ERβ and AR mRNA occurs in all germ cells during the mating period but in the experimental samples the spg and spcI are negative whereas spcII, spd and spz remain positive. A significant down-regulation of these receptors was also described in the gonadal tissue of the hermaphrodite *Rivulus marmoratus* exposed to NP (Seo et al., 2006). On the basis of our results, it may be hypothesized that in *Podarcis* the lack of AR and ER expression in spg and spcI of the NP-polluted samples could be responsible for the slowdown of spermatogenesis and failure in the cell replacement from the basis of the tubules. In wild samples our recent observations suggest that the frequency of spermatogenic waves may be regulated by the amount of spcI expressing both androgen and estrogen receptors (Verderame et al., 2014).

The epididymis runs parallel to the testis activity and plays a key role in fertility, ensuring sperm maturation and storage. Studies on the effects of NP on the epididymis are scarce and are conducted only in rats where gavage of NP leads to a significant reduction in the sperm number and motility (Uguz et al., 2009; Aly et al., 2012). Moreover, in the epididymal sperm of rat, NP reduces the integrity of the acrosome due to the high level of generated reactive oxygen species (Han et al., 2004).

From our results it emerges that in *Podarcis* treated with NP during the mating period, the cells lining the epididymal *corpus* are reduced in height and scattered secretory granules and very few or no sperm are present in the lumen, resembling the non-reproductive period. The absence of a large amount of sperm inside the epididymal channel may be closely related to lower spermatogenic activity in the testis affected by NP treatment. In addition, we know that the morpho-physiology of *Podarcis* epididymis around the annual cycle is regulated by the alternate expression of ERα whereas AR and ERβ are always expressed. In particular, in the epididymal *corpus* the expression of ERα naturally occurs in the post mating period, when the plasma levels of E_2 are at the zenith, and is absent during the mating period when the cells lining the *corpus* are highly secreting and the E_2 level is lowest. In the same way, this secretory activity may be inhibited by experimental administration of E_2 in the mating period and the expression of ERα elicited, as happens in the post-mating period (Angelini and Botte, 1992; Verderame et al., 2012; Verderame, 2014). Hence the expression of ERα in the epididymal *corpus* in NP-treated samples of *Podarcis* confirms once again the estrogen-like properties of NP.

Further investigations will be needed to clarify whether the alteration of the estrogenic environment during the mating period may interfere directly on the expression of steroid hormone receptors or indirectly by acting on the release of pituitary gonadotropins.

4.1. Conclusion

In conclusion, we showed that the ingestion of food and water polluted with an EDC with estrogen-like action, i.e., NP, affects the spermatogenesis and morpho-physiology of the epididymis during the mating period and interferes with the reproductive cycle the lizard *P. sicula*. At present, these results are the first on a terrestrial non-mammalian vertebrate and highlight what would happen if the animals were exposed to a polluted diet in a polluted environment. In addition, in the seasonal breeding species, a phase shift in the reproductive cycle opens up a number of issues related to the continuity of the species. Furthermore, and not least, given the widespread use of NP, possible frequent exposure could give rise to severe damage in the reproductive capacity even of humans.

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