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Using Radioimmunoassay to Investigate the Uptake by Amphibians of Estrogens Present in WWTP´S Effluent

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Abstract

The concern about health of aquatic fauna living in waters containing dynamic levels of estrogenic compounds has been increased in last years, in part responsible for disruption of reproduction and development of several wildlife populations, altering blood hormone levels, reducing fertility and fecundity, masculinisation of females and feminization of males. Low-level exposures also cause similar effects in human beings. However, the information of impacts of estrogenicity on wildlife populations is limited to a few species of animals, including some vertebrate and invertebrate.

The aim of this study was, therefore, to investigate the concentrations of estrone and 17β-estradiol in *Rana/Pelophylaxperezi*on four rivers downstream output´s waste water treatment plants, located in Portugal central region, comparing to two control groups living in a non-contaminated water environment. Concentrations between 53-101 pg/ml were founded for estrone, and 37-149 pg/ml for estradiol, using the radioimmunoassay method in blood/serum fraction of the amphibians.

Keywords: Estrone; 17β-Estradiol; Amphibians; RIA; Serum Fraction

Introduction

Endocrine Disrupting Compounds (EDCs) encompass a variety of chemical classes, including drugs, pesticides, compounds used in the plastics industry and in consumer products, industrial by-products and pollutants, and even some naturally produced botanical chemicals. Estrogens are chemical pollutants that can disrupt the endocrine system of animals by binding and activating the estrogens receptor(s). Large-scale efforts are now in progress to develop a better understanding of how these compounds disrupt physiological function; however the information on the impacts of these compounds on wildlife populations is limited to a small number of vertebrate and invertebrate species [1].

Many studies have shown that endocrine disruptors can cause adverse biological effects in animals, and low-level exposures also cause similar effects in human beings. The reports of increased number of cancer, and hormone-dependent decrease in the quantity and quality of sexual gametes in humans led to questions about the role of these compounds [2]. Upon insertion of chemical compounds into the environment, it became apparent the connection between environmental contamination of rivers and lakes and a wide variety of defects in the growth and reproduction in some species.

There is evidence that a wide variety of anthropogenic chemical compounds have properties and mechanism of similar action to endogenous hormones. Thus, estrogens in "environmentally relevant concentrations" are likely to cause morphological abnormalities, biochemical, physiological and behavioural responses of vertebrates and invertebrates [3].

The environmental research of these hormones consists in measuring concentrations in the effluents, testing the effluents toxicity in animals and evaluating the effects on animal and plant communities. Estrogens monitoring in the environment has become of great interest, mainly due to frequent detection in effluents of Waste Water Treatment Plants (WWTPs), waters for human consumption, rivers and lakes, at concentrations in the range of ng L-1 to μg L-1 [4]. Estrone (E1) and 17β-estradiol (E2) are natural female sex hormones produced by humans, mammals and other vertebrates. In order to assess the risk of exposure are some studies conducted in this area, and much research has been conducted in laboratory and field with various species of fish, reptiles, birds and mammals [5].

Amphibians have permeable skin, live in water and soil, and due to their physiology and life cycle, are exposed to certain environmental changes, are particularly sensitive to chemical pollution of aquatic systems, documented as one of the causes for the increasing extinction of these animals nowadays.

Therefore, the aim of this study was to identify the presence of estrone and estradiol in amphibians (only males), in their own wildlife, and thus, contribute with useful information for the conservation of these species of vertebrates.

Materials and Methods

Amphibians and sampling sites

The amphibians used in this study were *Rana/Pelophylaxperezi*, sexually mature male, medium size, body length between 80-100 g,

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body weight between 8-11 cm and with an approximate age of 3-6 years old. In the case of a species protected by international conventions (Berne Convention), national legislation and specific Community ("Habitats Directive"), a license was requested to the Institute of Nature Conservation and Biodiversity (ICNB) of Portugal to arrest temporally and also to collect the biological material of individuals. A total of 24 adult male *Rana* were captured between September and November 2011, four individuals per site/location.

Six sites were studied in four locations in Portugal central region, two control groups and four target groups in rivers, The four target groups are located at the Mondego River (GPS 40ºN 21.928; 8ºW 44.661), the Vouga River (GPS 40ºN 69.125; 8ºW 59.015), the Cértima River (GPS 40ºN 49.465; 8ºW 48.290) and the Arunca River (GPS 39ºN 91.343; 8ºW 62.961). All sites were situated downstream from WWTP´s outputs. The two control groups (Control Group I and Control Group II) were located in Coimbra in two domestic sources, with the GPS coordinates 40°N19.770; 8ºW 84.714 and 40°N20.931; 8ºW 84.173, respectively, located outside the influence area of Waste Water Treatment Plants (WWTPs)outputs.

Sampling procedures

The animals arrest was restricted to the time required for the manipulation of the individuals, to obtain a sample of biological material (blood) *in situ*. The capture of the individuals was performed using dip-nets.

After capture, the amphibians were anaesthetized with chloroform from Sigma-Aldrich, and blood collected via leg vein using insulin syringes. After this procedure, blood collection was transferred into a pre-coagulation tube. All blood samples were centrifuged at 5000*g* for 10 min and refrigerated at 4ºC, to separate out the serum fraction. Serum was then introduced into 1 mL vials and stored at −20ºC prior to measurement by RIA.

Serum estrogens analysis

Double AntibobyEstradiol^{125}|radioimmunoassay (PIKE2D-6), from Siemens Medical Solutions Diagnostics was used. This kit of estradiol is a double antibody radioimmunoassay sequential procedure in which the sample is pre-incubated with antibody-anti-estradiol. The ^{125}|labeled estradiol competes with estradiol in the sample for binding sites to the antibody. In order to obtain the calibration curve for E2 seven standards with concentrations of 0, 5, 10, 20, 50, 150 and 500 pg mL^{-1}were used. 200 µl of standards or samples was introduced into each tube together with 100 µl of E2 antibody, shacked using a vortex, and incubated for 2 hours at room temperature. Then, 100 µl of labelled ^{125}I E2 was added to all tubes and incubated for 1 hour at room temperature. After incubation, the separation of bound and free^{125}I E2 is performed by the double antibody method accelerated by polyethylene glycol (PEG), thus a 1mL of cold precipitating solution was added to all tubes and agitated in a vortex, followed by 10 min of incubation at room temperature. Precipitating solution consisted of goat anti-rabbit gamma globulin and diluted polyethylene glycol in saline solution. Tubes were then centrifuged for 15 min at 3000 *g*, the liquid phase was removed and the precipitate retained for counting. Each tube was counted for 60 seconds.

Estrone-RIA-CT (KIPI9100) *in vitro* diagnostic used was the Radioimmunoassay for the Quantitative Determination of Estrone in Human Serum or Plasma, *in vitro* diagnostic use, from DIA source Immuno Assays S.A. In order to obtain the calibration curve for estrone, seven standards with concentrations of 0, 12.5, 25, 50, 125, 250 and 750 pg mL^{-1}were used. For each tube 100 µl of standards or samples was pipetted. After, 400 µl of labelled ^{125}I Estrone was added to all tubes, agitated using a vortex and incubated for 2 hours at room temperature. After incubation, 2 ml washing solution (buffered solution containing sodium azide< 0.1 %) was added and then the liquid phase was decanted. This washing procedure was repeated two times. The radioactivity was counted in each tube for 60 seconds.

Results and Discussion

Calibration curves

The data values were obtained using different concentrations of 17β-estradiol (Table 1) and of estrone (Table 2).

The experimental values for standards were fitted to a four-parametric logistic equation (4PL) [6]:

$$Y = [(A - D)/ [1+(x/C)^B]] + D \quad \text{Eq 1}$$

where:

Y- response value, x - antigen concentration, A - zero standard concentration, B- slope factor, C- concentration value at the inflection point, D- standard concentration excess.

However, in order to compare several standard curves, the Ydata should be normalized between 100 % (A) and 0% (D), according to the ratio:

$$Y_N = (Y - D)/(A - D)*100 \quad \text{Eq 2}$$

Where Y_N is the normalized curve response, A and D are parameters of the 4PL [7]. Sensitivity can be expressed by the limit of detection (LOD), generally considered as the lower concentration that produces a signal significantly different from the blank signal [6].

Standards	pg mL^{-1}	Mean CPM*	B/B0 %**
A	0	7609,6	100
B	5	6054,7	79,56
C	10	5478,2	71,90
D	20	4697,9	61,73
E	50	3740,8	49,16
F	150	2658,7	34,90
G	500	1528,6	20,08

*Counts per minute
**Normalized response

Table 1: Results obtained for 17β -estradiol standards.

Standards	pg mL^{-1}	Mean CPM*	B/B0 %**
A	0	14535,7	100
B	12,5	12113,0	92,50
C	25	11191,2	81,90
D	50	9400,2	70,30
E	125	6441,6	52,60
F	250	4295,0	37,80
G	750	2029,8	17,90

*Counts per minute
**Normalized response

Table 2: Results Obtained for Estronestandards.

The calibration curves for the two estrogens were considered satisfactory, with correlation coefficients for each estrogen of 0.999 (Table 3). So, this technique was used to determine the concentration of estrogen in amphibians.

Quantification of estrogens on serum samples

The levels of estrogens in each sample were measured in triplicate, after the experimental procedure described in Double AntibobyEstradiol^{125}|radioimmunoassay (PIKE2D-6), (volume of 200 µL) and described in Estrone-RIA-CT (KIPI9100) (volume 100 µL). After the counts per minute of each sample were converted to pg/mL using the calibration curves and results are presented in Graph 1.

When analyzing the values of the concentrations of estrogens found in 24 frogs studied, levels of estradiol recorded are higher than those observed for estrone in the experimental groups I, II and control III. The reverse is the case in groups II, IV and control group II (Graphs 1 and 2).

Comparison ofresults obtained between experimental and control groups

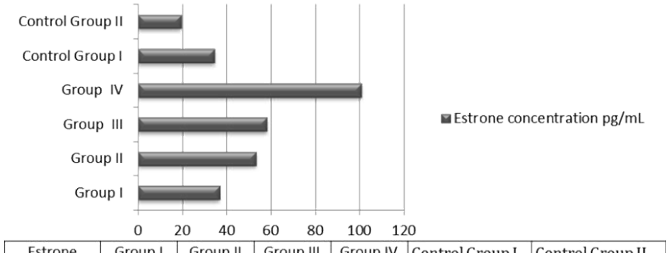
In literature, no studies were found with similar samples, so we proceeded to compare the results obtained between the experimental groups and control group I and control II, using the Kruskal-Wallis test. A further comparison between pairs of groups (Table 4) was performed with the U test of Mann-Whitney. P values <0.05 were considered statistically significant. For statistical analysis the Statistical Package for Social Sciences was used, Windows version (SPSS 18.0).

For estradiol there is a predominance of higher values in the experimental groups compared to control group I, with statistically significant differences (p=0.001). Comparing pairs of groups surrounding each experimental group and control group I, differences in values remain significant whatever the compared pair (p <0.05). When the control group II used is not always the predominance of higher values observed in the experimental groups. In particular the groups II and IV show lower values than those recorded in the control

	A	B	C	D	Correlation coefficient
Estrone	14289	1,06	75,88	1568	0.999
SD*	±476,16	±0,19	±15,93	±974,79	
Estradiol	7674	0,58	46,52	14,63	0.999
SD	**±71,16**	**±0,03**	**±7,76**	**±355,25**	

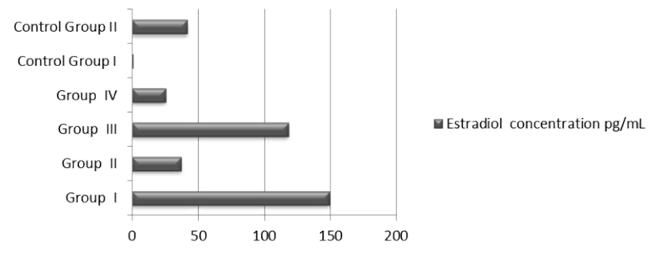
*SD = standard deviation

Table 3: Parameters values of four-parametric logistic equation (4PL) for estrone and estradiol (n=3).



Estrone	Group I	Group II	Group III	Group IV	Control Group I	Control Group II
Conc pg/mL	36,75	53,31	58,16	100,98	34,52	19,34
SD	± 0,179	± 1,259	± 0,816	± 0,872	± 2,216	± 1,359

Graph 1: Characterization and concentrations of estrone found in Rana/ Pelophylax perezi, n=3.



Estradiol	Group I	Group II	Group III	Group IV	Control Group I	Control Group II
Conc pg/mL	149,43	37,22	118,1	25,31	0,037	41,79
SD	± 1,249	± 0,199	± 0,132	± 0,003	± 0,022	± 0,877

Graph 2: Characterization and concentrations of estradiol found in Rana/ Pelophylax perezi, n=3.

	Estradiol (pg/ml)	p†	Estrone (pg/ml)	p†
Experimental group I	149,431		36,750	
Experimental group II	37,221		53,310	
Experimental group III	118,103		58,160	
Experimental group IV	25,312	0,001	100,98	0,001
Control group I	0,037		34,519	
Control group II	41,798		19,339	

† Kruskal Wallis Test

Table 4: Comparison between experimental groups and control groups.

group. The comparisons between all the groups involved register value significant (p=0.001) and remained significant during the comparison between each of the experimental groups and control group II (p <0.05).

For estrone the statistical significance remains to compare the various groups involved, equally with higher values for the experimental groups. A further comparison between pairs of groups reveals that these differences are significant when analysing groups II to IV and the control group I (p<0.05). In the pair experimental I/ control I the p that was observed was not significant, for these estrogen, the statistical significance remains for the comparison between the different groups involved, also with greater magnitude values for the experimental groups, with significant differences either in the comparisons performed for all the groups (p= 0.001) or in comparison of pairs of groups (test I, II, III and II versus the control group, p <0.05).

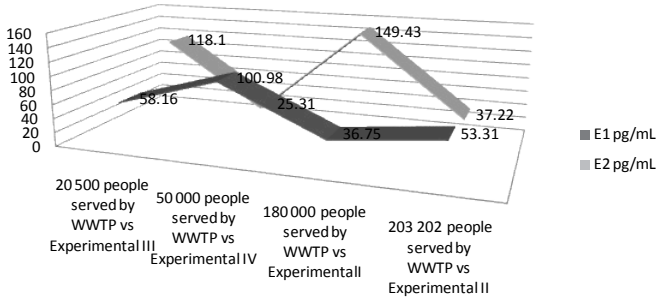
Since the concentration of estradiol levels reported are higher than those observed for estrone in the experimental groups I and III and lower in groups II and IV, this data was compared with the number of inhabitants served by the WWTP, as shown in Graph 3.

For estrone there are higher values for the effluent of WWTPs who serving a number of 20.500 people and 50.000 people, there is a predominance of higher values in the effluent of the WWTPs who serving 20.500 peoples and 180.000 for estradiol.

According to the presented results is evident the persistence of estrogen concentrations in WWTPs output´s. This indicates inefficiency in removal of these compounds during water treatment, which are systematically discharges to the rivers, lakes and seas, affecting ecosystems and drinking water sources.

Conclusions

This study provides evidence that prolonged exposure to the



Graph 3: Comparison between pairs of experimental groups and the number of people who are served by the WWTPs effluent (rivers).

WWTP effluent may cause cumulative effects of estrone and estradiol in males of the green frog *Rana/Pelophylaxperezi.* Among the various studies in vertebrates and invertebrates described in the literature, most were made in the laboratory where the target populations were exposed to concentrations of estrogens which revealed some changes, like altering blood hormone levels, reducing fertility and fecundity, masculinisation of females and feminization of males [8-11]. In other work, carried out in situ, the animals were exposed to environmentally relevant concentrations, and it was verified that, for example, there were sex changes in several species of fish, lack of reproduction in birds and abnormalities in the reproductive organs of alligators and polar bears [12]. Many adverse effects are described in different studies in several species of vertebrates and invertebrates (less studied) [5,8,10,13-15].

Due to the lack of reference values for the concentration of estrogen in these individuals probably the effects are comparable to the ones observed and described in several studies in vertebrates, such as feminization, male infertility, etc., possibly resulting in an effect at the level of the population. We can, however, infer that there are cumulative processes of estrogens in these individuals, who have their natural habitat in freshwater environments in the studied rivers where the WWTPs effluents are released, with obvious potential estrogenic activity, and probably in the drinking water from rivers.

These animals, by their natural physiology, living in water, have been continuously exposed to chemicals present in aquatic systems, so is also important say that the identified estrogenic activity could be a possible cause for serious global decline of many amphibian species and the increased risk of extinction of these animals.

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