UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA
CÂMPUS DE BOTUCATU

ABORDAGEM METODOLÓGICA PARA AVALIAÇÃO DO POTENCIAL DESREGULADOR ENDÓCRINO DE ÁGUA DE BEBER - ESTUDO COM AMOSTRAS REAIS

MARIZE DE LOURDES MARZO SOLANO

Tese apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista – UNESP como parte dos requisitos para obtenção do título de Doutor em Patologia.

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Ó mar salgado, quanto do teu sal
São lágrimas de Portugal!
Por te cruzarmos, quantas mães choraram,
Quantos filhos em vão rezaram!
Quantas noivas ficaram por casar
Para que fosses nosso, ó mar!

Valeu a pena? Tudo vale a pena
Se a alma não é pequena.
Quem quer passar além do Bojador
Tem que passar além da dor.
Deus ao mar o perigo e o abismo deu,
Mas nele é que espelhou o céu.

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Contaminantes ambientais e interferentes endócrinos

Existem mais de 11 milhões de substâncias químicas registradas no Chemical Abstracts Service (CAS), sendo só os Estados Unidos responsáveis pelo registro de 1200 a 1500 novas substâncias por ano[1]. A produção e uso crescente de compostos químicos, e a ineficiência de políticas de controle e/ou tratamento, têm levado ao aparecimento dos chamados contaminantes emergentes do ambiente. Entre outros efeitos potencialmente adversos, esses contaminantes podem interferir no sistema endócrino de seres humanos e de outros animais, afetando seu crescimento e reprodução [2]. Conhecidas como interferentes endócrinos (IE), essas substâncias são agentes químicos individuais ou misturas capazes de mimetizar hormônios naturais ou inibir seu funcionamento, comprometendo o sistema endócrino e, consequentemente, prejudicando a saúde de um indivíduo, de sua prole e de toda uma (sub)população [3-5]. Os IE, na sua maioria são derivados de químicos antropogênicos, provenientes de pesticidas, plásticos, detergentes, tintas e outros compostos industriais ou domésticos [3]. Em geral, pertencem ao grupo dos poluentes orgânicos persistentes (POPs) e, como tal, têm dispersão ambiental rápida e vasta pela cadeia alimentar [3, 6]. Os IE podem ser agrupados em classes de substâncias: as naturais e as sintéticas. A classe de substâncias sintéticas inclui agroquímicos, como inseticidas, herbicidas, fungicidas, bem como substâncias utilizadas nas indústrias, como dioxinas, bifenilas policloradas (PCB), alquilfenóis, hidrocarbonetos aromáticos policíclicos (HAP), ftalatos, bisfenol A e metais pesados, além de compostos farmacêuticos, como os estrogênios sintéticos dietilestilbestrol (DES) e 17α-etinilestradiol. Na classe de substâncias naturais

destacam-se os fitoestrogênios, como a genisteína, o metaresinol, e os estrogênios naturais, como 17β-estradiol, estrona e estriol [6, 7].

Ocorrência de IE no ambiente

A fragilidade das políticas públicas e a precariedade dos serviços de saneamento no país, somadas ao crescimento populacional desordenado nas grandes cidades, têm sido os principais responsáveis pela diminuição da qualidade das águas de rios, lagos e reservatórios. Além disso, a escassez de recursos financeiros e a inexistência de planejamento baseado em critérios toxicológicos e ambientais conduzem a um quadro em que o lançamento de esgoto doméstico não tratado, junto com descargas industriais, causam sérios impactos aos sistemas de águas superficiais [8-10].

Devido à importância toxicológica humana e ambiental dos IE é de extrema importância elucidar suas ocorrências nos sistemas aquáticos. Particularmente, as águas superficiais são matrizes ambientais complexas devido sua composição química, e diversos compostos orgânicos presentes necessitam ser avaliados. Mesmo em países desenvolvidos, muitos estudos têm evidenciado a ocorrência desses compostos também em sistemas de águas superficiais [11-17].

Resultados recentes mostraram atividade estrogênica, embora pequena, de amostras de água para consumo humano coletadas no Estado de São Paulo e testadas em um bioensaio *in vitro* com linhagens modificadas de *Saccharomyces cerevisiae.* Essa levedura modificada é capaz de responder a agentes estrogênicos devido a existência de um inserto do gene que codifica o ER humano [18, 19]. Esses resultados sugerem que a população humana pode estar exposta a uma classe de compostos cujos efeitos

somente agora vêm sendo reconhecidos. Ainda são desconhecidos os níveis seguros de IE em água potável, sendo recomendável que estas substâncias sejam investigadas e monitoradas a fim de permitir ações preventivas para minimizar as fontes e os níveis de exposição. Para isto, naturalmente, o primeiro passo é verificar se a água tratada fornecida ao público exerce efeito estrogênico em sistemas mais complexos que os de leveduras, como são os mamíferos.

Sistema endócrino

Em mamíferos, moléculas endógenas como hormônios esteróides, da tireóide, retinóides, vitamina D, e outros, se difundem pela membrana plasmática das células alvo ou são produzidas pela própria célula e se ligam aos receptores nucleares (NR)- uma classe de proteínas encontradas no interior das células. Os efeitos estrogênios predominantes são mediados por dois NR distintos: receptor de estrógeno alfa (ERα) e beta (ERβ). Estes são membros de uma superfamília de receptores ligante-dependentes de fatores de transcrição [20]. Então, após ligação e ativação do NR, ocorre ligação específica do receptor com elementos responsivos do DNA na cromatina nuclear e o recrutamento de uma variedade de cofatores que modificam a organização cromossomal. Estes cofatores então regulam a expressão de múltiplos genes através de comunicação com o aparato transcricional, alterando a taxa de transcrição dos genes alvo [21]. Portanto, a ligação com NR é apenas o primeiro passo na cadeia de eventos que leva a alterações na expressão gênica e subsequente sinalização celular [22]. Há grande variação no número de ER, coativadores e corepressores expressos nos diferentes tecidos, e é por isso que estrógenos têm múltiplos efeitos fisiológicos, não apenas no sistema reprodutivo, mas também em outros órgãos como

o fígado, ou mesmo sistemas inteiros como os sistemas nervoso, cardiovascular, muscular, esquelético, e o imune [23, 24].

Para o entendimento dos mecanismos de ação e das consequências da exposição aos IE, uma série de características do sistema endócrino devem ser consideradas. Essas substâncias podem agir em baixas doses através de uma grande variedade de mecanismos, incluindo processos dependentes e independentes de receptores, espécie animal, tecidos e células e metabolismo [25]:

- mimetizar efeitos dos hormônios endógenos (como o estradiol) pela ligação com o receptor desses hormônios;
- antagonizar efeitos dos hormônios endógenos por bloqueio da ligação entre o hormônio e o receptor (como o tamoxifen);
- reagir direta/indiretamente com a estrutura do hormônio endógeno, alterando-o;
- interferir na síntese dos hormônios endógenos;
- alterar a quantidade de receptores hormonais disponíveis.

Os IE mais conhecidos têm atividade estrogênica, sendo por isto denominados xenoestrógenos [25]. Historicamente, o foco nos xenoestrógenos como desreguladores das funções reprodutivas na espécie humana surgiu após o reconhecimento da carcinogenicidade do dietilestilbestrol (DES), usado em larga escala como antiabortivo entre 1947 e 1971 [2]. De 1970 a meados de 1990, ficou progressivamente mais claro que os níveis elevados de poluição ambiental afetavam espécies silvestres como peixes, aves, répteis, etc, [26-28] levando ao desenvolvimento de hermafroditismo, masculinização/feminização [29] e outras desordens reprodutivas [30].

Regulação neuroendócrina

A hipófise anterior controla a atividade de glândulas endócrinas pela secreção de hormônios tróficos, os quais são controlados por *feedback*, dependendo da concentração do hormônio primário na circulação, e também por fatores de liberação secretados pelo hipotálamo. O eixo hipotalâmico-hipofisário-gonadal (HPG) é parte crítica no desenvolvimento e regulação do sistema reprodutivo e controla o desenvolvimento e reprodução do homem e dos animais. O hipotálamo produz o hormônio liberador de gonadotrofina (GnRH), que se liga aos receptores das células secretoras da adenohipófise anterior [31], as quais secretam o hormônio luteinizante (LH) e o hormônio folículo estimulante (FSH), que ativam os ovários à produzir estrógeno e inibina, além de regular o ciclo de crescimento uterino e amadurecimento dos folículos ovarianos [32]. O estrógeno também induz o *feedback* negativo no eixo HPG, pela inibição da produção de GnRH no hipotálamo. Flutuações dos hormônios hipofisários causam alterações dos hormônios produzidos pelas glândulas alvo do sistema reprodutor, o que acarreta efeitos locais e sistêmicos no organismo. Na fase pré-púbere de animais e humanos o eixo HPG ainda é imaturo e não exerce controle hormonal adequado. Níveis elevados de FSH plasmático em ratas imaturas, por exemplo, parecem ser em parte decorrentes de relativa ineficiência de *feedback* estrogênico negativo nessa idade [33, 34]. Por outro lado, os níveis de LH, embora às vezes também elevados durante o desenvolvimento, mostram maior variação individual, um fenômeno que tem sido interpretado como liberação tipo pulsátil de LH [33]. Tais “picos” de LH têm sido postulados como decorrentes da ação *feedback* positiva do estrógeno na idade pós-natal e pode ser um evento frequente nessa fase [35]. O aumento dos

níveis de gonadotrofinas (LH e FSH) é posteriormente (puberdade) diminuído pela ação inibitória da prolactina e/ou progesterona [33].

Interessante lembrar que existem formas de interação dos IE em órgãos como a tireóide, importante na regulação do metabolismo funcional de muitos outros sistemas do corpo. O hormônio estimulante da tireóide (TSH), bem como o FSH e o LH, é uma glicoproteína de cadeia dupla, reguladora da função e secreção da tireóide. A produção desse hormônio é pela hipófise, e o controle por *feedback* negativo ocorre pelos níveis circulantes de tiroxina (T4) [36]. Os efeitos de T4 são mediados via hormônio triiodotironina (T3) (T4 é convertido em T3 em tecidos alvo), três vezes mais potente que T4 [37].

Esteroidogênese

IE podem também atuar nos receptores de membrana, receptores neuronais pré-sinápticos, receptores órfãos, e vias enzimáticas envolvidas na síntese de hormônios. Enzimas esteroidogênicas são responsáveis pela biosíntese, a partir do colesterol, de vários hormônios esteróides, incluindo glucocorticóides, mineralocoritcóides, progestágenos, andrógenos e estrógenos. Consistem de várias enzimas específicas, como o citocromo P450 (CYPs), hidroxisteróide desidrogenases (HSDs) e esteróide redutases[38].

Vários órgãos são conhecidos pela sua capacidade de sintetizar esteróides biologicamente ativos, incluindo as glândulas adrenais, testículos, ovários, cérebro, placenta e tecido adiposo. No ovário, o folículo antral e o corpo lúteo são glândulas esteroidogênicas, cujas células da granulosa e da teca trabalham juntas para produzir esteróides [39]. Dentre as formas de estrógenos na reprodução feminina, o estradiol (E2)

é o estrógeno mais potente, isto é, com maior eficácia e afinidade nas ligações com os NR, seguido pela estrona (E1) e pelo estriol (E3).

Particularmente, a aromatase (CYP19), enzima que converte andrógenos em estrógenos, tem sido objeto de estudos sobre os mecanismos pelos quais compostos químicos interferem na homeostase e função dos hormônios esteróides sexuais, geralmente relacionados aos processos de (de)feminilização e (de)masculinização [40]. A CYP19 é expressa principalmente no cérebro e gônadas, mas é também encontrada na placenta e nos tecidos adiposo e ósseo [41]. A aromatase cataliza as etapas finais da biossíntese de estrógenos, convertendo androstenediona em estrona, e testosterona em estradiol. Em mamíferos, a diferenciação em fenótipo masculino depende não somente da testosterona, mas também do estradiol gerado a partir da testosterona pela aromatase. Dessa forma, distúrbios na expressão da aromatase e/ou alterações na sua atividade catalítica exercem efeitos negativos, como hipoestrogenismo e virilização feminina, ou puberdade precoce e câncer mama em mulheres e ginecomastia em homens[42]. Além disso, enzimas responsáveis pela síntese e metabolismo dos hormônios esteróides sexuais ovarianos (CYPs-11a1, 17a1, 19a1, HSD17B, entre outras), podem ter expressão e atividade catalíticas alteradas, quando são alvos de agentes químicos [39].

Potência e efeito dos IE

Os IE possuem potência de ação hormonal variada, podendo funcionar a baixas doses de maneira tecido-específica, ou apresentar respostas não lineares devido à dinâmica complexa de ocupação e saturação dos receptores hormonais. Então, as baixas doses podem, por vezes, induzir maior impacto num tecido alvo, e os efeitos e a

curva dose-resposta pode ser inteiramente diferentes quando comparado com altas doses [43].

Na avaliação de estrogenicidade, a verificação de alterações da expressão gênica induzidas por estrógenos é mais sensível que parâmetros clássicos, como determinação de níveis hormonais e aumento de peso uterino [44]. Alguns genes alvo de IE são o Hoxa-10 e o Calbindina –D9k: o Hoxa-10 é membro de uma família de fatores de transcrição essenciais para o desenvolvimento do trato reprodutivo, principalmente fertilidade feminina, sendo a progesterona seu indutor primário [45]; já o gene Calbindina–D9k (CaBP-9k) é membro de uma grande família de proteínas intracelulares que se ligam ao cálcio [46] e, no útero do rato, é estimulado pelo 17-β-estradiol. O Hoxa-10 é afetado pelo bisfenol A (BPA), metoxiclor e dietilestilbestrol (DES) após exposições *in vivo* e *in vitro* [45, 47, 48]. A expressão do CaBP-9k é rápida e fortemente induzida no útero de mamíferos por compostos estrogênicos ambientais como o DES [49]. Técnicas como a PCR em tempo real e *microarrays* têm sido utilizadas para determinar mudanças na expressão gênica e prever atividade estrogênica por IE. Essas técnicas permitem avaliar aumento ou diminuição da expressão, além de deleção e inserção de genes envolvidos em processos bioquímicos particulares, como são as respostas de órgãos/tecidos expostos à substâncias químicas com propriedades terapêuticas ou tóxicas [50].

Modelos experimentais

Em resposta à preocupação pública, agências governamentais e transnacionais, como a Agência de Proteção Ambiental Americana (USEPA), Agência Americana de Administração de Alimentos e Fármacos [51] e a Organização para Cooperação e

Desenvolvimento Econômico (OECD), estão analisando estratégias para identificar agentes químicos que têm potencial de interferência endócrina. É importante salientar que os ensaios atualmente disponíveis não conseguem abranger todos os efeitos sistêmicos provocados por esses agentes [52-55]. Dentre o grande número de ensaios *in vivo* e *in vitro* disponíveis, o ensaio uterotrófico é proposto pela OECD como um teste de triagem *in vivo* para detectar atividade estrogênica [25, 53, 56]. Ele consiste em quantificar modificações de peso uterino em ratas imaturas ou ooforectomizadas após tratamento com a substância teste por período de 3-4 dias [57, 58]. Além desses parâmetros, embora basicamente qualitativo, podem ser analisados o epitélio vaginal (teste de cornificação vaginal), que também tem sido utilizado para detectar mudanças dependentes da xenoexposição [25], e o endométrio[53]. Outro órgão alvo importante utilizado nos ensaios harmonizados adotados pela USEPA, USFDA e OECD para identificação dos IE em roedores é o ovário, onde esses agentes podem agir sobre folículos maduros e imaturos, levando à infertilidade temporária ou permanente [59, 60-56]. Ensaios mais completos como o de desenvolvimento prepuberal são capazes de detectar IE que atuam por uma grande variedade de modos de ação: compostos (anti)estrogênicos e (anti)androgênicos fracos e fortes; inibidores da biossíntese de esteróides; inibidores de aromatase; inibidores da 5α-redutase; agentes ativadores da tireóide, e compostos que afetam os eixos hipófise-pituitária-gônada e hipófise-pituitária-tireóide [61]. Alguns desses modos de ação não são detectados por ensaios *in vitro* e mesmo alguns *in vivo*, o que torna um resultado positivo nesse ensaio de desenvolvimento prepuberal mais confiável [22].

Além do programa ToxCast da USEPA (http://www.epa.gov/ncct/toxcast/) e REACH da Comissão Européia (http://ec.europa.eu/enterprise/sectors/chemicals

/reach/) para detectar mais rapidamente substâncias tóxicas e possíveis IE, recentemente também foram criados modelos computacionais e base de dados para prever a complexa relação concentração-duração-resposta que pode ocorrer nos ensaios *in vivo*. Breen e colaboradores (2013) [62] desenvolveram um modelo computacional do eixo hipotalamico-pituitário-gonadal de fêmeas de peixe *Pimephales promelas* para prever a resposta adaptativa desse sistema a inibidores de aromatase. Também, um outro grupo reuniu uma base de dados de acesso livre chamada EADB (Estrogenic Activity Database), que permite tanto cientistas como a comunidade regulatória verificar e avaliar o potencial estrogênico de substâncias químicas [63]. Esses estudos *in silico* representam uma evolução em relação ao modelos experimentais para examinar e prever o comportamento dinâmico de sistemas perturbados, além de fornecer importantes ferramentas para a avaliação do risco.

Assim, considerando todos os pontos abordados, optamos por investigar efeitos de interferência endócrina, toxicidade reprodutiva e alteração da expressão gênica em ratos Wistar fêmea expostas a extratos concentrados de amostras de água para consumo humano de uma determinada Estação de Tratamento de Água. Para isso, delineamos um projeto que aborda protocolos internacionalmente utilizados na regulação de novos compostos, mas modificados com a inclusão de outros parâmetros mais sensíveis e de alta tecnologia, como apresentados no manuscrito no Capítulo II.

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A METHODOLOGICAL APPROACH TO EVALUATE ENDOCRINE DISRUPTING ACTIVITY OF DRINKING WATER – REAL SAMPLES STUDY IN RATS

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Abstract

If contaminated waters are treated by conventional processes (WTP) emerging contaminants are not fully removed and will remain in the drinking water (DW). We investigated the potential for endocrine disruption of two DW samples collected in 2010 and 2012 from a WTP located in São Paulo, Brazil. Water samples were extracted and chemically analyzed for emerging substances and evaluated with an *in vitro* bioluminescent yeast assay containing estrogen/androgen human receptors (BLYES/BLYAS). *In vivo* assays were conducted with 21-day old female rats exposed to DW extracts for 03 (uterotrophic assay; OECD) or 20-days (pubertal assay, EPA). The exposure represented a daily ingestion of 2 L, 5 L and 10 L of DW by a 60 kg human being. Caffeine (5.8 - 21 ug/L), estrone (1 ng/L), atrazine (2.2 -11.2 ng/L), carbendazim (0.22 ng/L), azoxistrobina (0.23 ng/L) and tebuconazole (0.19 ng/L) were detected in DW by LC-MS/MS-ESI. There were no increase of the uterus wet weight in the uterotrophic assay, and no alteration of the vaginal opening moment in the pubertal assay. However, there was increased relative blotted uterus weight in animals treated for 3-days with both DW. Levels of LH and FSH presented a significant dose-response increase in the uterotrophic assay developed with the 2010 sample. This happened in association with a significantly increased incidence of vaginal keratinization after the 3-day exposure. Results suggest the DW tested exerted hypothalamic-hypofisis activity alteration *in vivo.* Additional end-points in standard protocols could be useful to evaluate the presence of endocrine disruptor activity in DW.

Keywords: drinking water, emerging contaminants, endocrine disruption, yeast assay, uterotrophic rat assay, prepubertal female rat development assay.

Abbreviations: BLYES, bioluminescent yeast assay with human estrogen receptor; BLYAS, bioluminescent yeast assay with human androgen receptor; DW, drinking water; ED, endocrine disruptors; EPA, U.S. Environmental Protection Agency; E1, Estrone; E2, 17-β estradiol; E3, Estriol; FSH, Follicle-Stimulating Hormone; LC-MS/MS-ESI, Liquid Chromatography Tandem

Mass Spectrometry; LH, Luteinizing Hormone; OECD, Organisation for Economic Co-operation and Development; P4, Progesterone; PRL, Prolactin; T, Testosterone; VO, Vaginal Openning; WTP, Water Treatment Plants;

1. Introduction

The release of raw or treated sewage into surface waters has drawn attention to an important current issue: the water quality. Many xenoestrogens, as well as other chemical substances, may remain even after drinking water (DW) treatment conventional processes [1, 2]. In Brazil, many studies confirmed that sewage discharges, with or without previous treatment, are responsible for the occurrence of estrogens and xenoestrogens in waters [3-5]. São Paulo State has only 50% of its sewage collected and treated. As a consequence great amounts of different classes of chemicals (pharmaceuticals, personal care products, hormones, drugs of abuse, pesticides, industrial chemicals) have been found in surface and DW samples [6-9]. Persistent compounds in the environment may cause adverse effects both in humans and biota even at low concentrations [10-13]. These include some natural estrogens like estradiol (E2), estrone (E1) and estriol (E3), together with the synthetic estrogen ethinylestradiol, and xenobiotics such as bisphenol A and alkylphenols which may induce endocrine alterations in humans and wildlife at ng/L levels. Another frequent contaminants, known to posses endocrine disrupting activities are the triazines such as atrazine, imidacloprid and fungicides like carbendazim, azoxistrobin and conazoles [9]. As an overall consequence, humans are exposed to several estrogens, anti-estrogens, androgens, anti-androgens and steroidogenic substances during their whole life and this has stimulated interest in assessing the effects associated with simultaneous exposure to these chemicals [14, 15].

Given the importance of these substances for humans and wildlife, is extremely important to elucidate the possible consequences of the occurrence of these endocrine disruptors in the

aquatic environment. Although the available literature deals with local industrial/domestic effluents and rivers quality characterization, the scenario of endocrine disruptors occurrence is global. Then, even in developed countries with high index of sewage treatment, the occurrence of estrogenic compounds deserves attention: natural and synthetic hormones are significantly elevated in European and USA rivers, for example [16-19], and the levels of alkyphenols, such as nonylphenol, octylphenol and bisphenol A range from ng/L to ug/L in many surface waters around the world [19-22]. These observations indicate that environmental endocrine-disrupting compounds can eventually be found in DW, leading to direct human exposure.

The objective of this investigation was to evaluate the potential for endocrine disruption of DW samples collected at a Water Treatment Plant (WTP) of São Paulo, Brazil using treated water extracts for the evaluation of the prepubertal female rat endocrine system. Screening tests such as *in vitro* androgen and estrogen human receptors response assay (BLYES/BLYAS assays) were also conducted and more sensitive *in vivo* end-points were added to improve the robustness of standard assays for detecting the potential endocrine activity of mixtures of poor data chemicals at low doses. This experimental design also represents a contribution to the methodological approach to evaluate endocrine disrupting potential of DW samples.

2. Results

2.1 Chemical analyses and bioluminescent BLYES/BLYAS assays

Seven of the 31 compounds investigated, atrazine, caffeine, estrone, carbendazim, imidacloprid, azoxistrobin and tebuconazole, were detected in one or both DW samples (Table 1). The last four compounds were not evaluated in the 2010 sample due to technical difficulties at the time. The differences between the two water samples tested are small and referred mainly to the amounts of caffeine or atrazine, which were almost 104 or 14 fold

higher in the 2010 sample, respectively. We also found 1ng/L of estrone in the 2010 sample, but not in the 2012 one.

No estrogenic nor androgenic activity was obtained *in vitro* when the yeast bioassay BLYES/BLYAS was applied (Table 1).

2.2 *In vivo* assessment

2.2.1 Uterotropic bioassay

2.2.1.1 In-life parameters and organ weights

In the most recent water collection sample, the 2012 DW extracts had no effect on food or water consumption, body or organ weights, and induced no clinical signs (data not shown). Otherwise, the 2010 water extract induced a significant decrease in food intake, not in a dose response manner, but reflected in a significant final body weight reduction, especially in the highest extract treatment group. Even so, the body weight gain was reduced but not significantly at the end of the experiment (not shown). We observed no differences in uterine wet weight gain among the treated groups. Blotted uterus weights increased with the increase of the water extract's doses, but it was just statistically significant for the absolute blotted uterus of the immature rats exposed to the middle and highest 2010 water sample dose levels (Table 2). The overall data on this parameter show that despite not always statistically significant, there was a uterus weight increase in the extract-treated groups when compared to the negative control. The positive control with 17-α-ethynylestradiol worked properly with an increased uterus wet weight response.

2.2.1.2 Histology and morphometry of reproductive organs

Histology of collected organs was unaltered after the water extracts exposure, but a significant increase of the endometrial thickness was registered in animals exposed to the 2010 water extract middle dose (Table 2).

There was no VO before the day of necropsy. However, there was a significant increase in the incidence of keratinization of the vaginal epithelium after exposure to the highest extract dose water collected in 2010 (Figure 2).

Primordial and primary ovaries follicles count per area (pooled together) was reduced in the middle and the highest 2010 water dose sample (Supplementary data), especially the primordial and primary follicles (not shown).

2.2.1.3 Hormone measurements

The hormone levels of both water samples were similar. The 2012 water sample results are in the Supplementary data. The 2010 water samples results are presented in Figure 3. LH and FSH levels were increased by treatment with the highest dose of 2010 water sample, but these levels were not justified by the estradiol or estrone levels, which were unchanged in the same animals. There were also no clear differences between groups when analyzing progesterone and prolactin concentrations. Testosterone was also decreased but not significantly after treatment with the both DW samples extracts.

2.2.1.4 Gene transcripts

No clear changes in the uterine transcript levels due to DW treatment from 2010 collection were recorded when investigating the expression levels of the estrogen receptors (ER-α/β), progesterone receptor (PR) and of endocrine disruptors target genes HOXa-10 and Calbindin-9k. Although not statistically significant, there were a decrease in the HOXa-10 and an increase

in the Calbindin-9k genes expressions of treated groups. Results are presented in Supplementary data.

2.3.1 Pubertal development and thyroid function in intact juvenile/peripubertal female rats

2.3.1.1 In-life parameters and organ weights

The 2010 DW sample extract had no effect on food or water consumption, but body weight gain was significantly reduced in 10% and 8% in the two highest dose groups, respectively. The liver absolute and relative weights were also reduced in the first and second treated groups (Supplementary data).

2.3.1.2 Histology, vaginal opening and estrus cyclicity

Histology of thyroid, uterus, ovaries, vagina, liver and adrenals were not altered after the 2010 water extract exposure. In contrast to the uterotropic assay, there were no alterations in any class of ovarian follicles of these animals treated with the same water extract samples.

Female pubertal parameters were not affected by DW extracts: there was no clear change in the age at VO, body weight at VO, mean age at first vaginal estrous and mean cycle length. The percentage of animals cycling at the end of experiment was 100% in all groups, except one female at the highest water extract dose that stopped cycling. Otherwise, there was also a decreased percentage of treated females regularly cycling, especially those exposed to the 2010 water dose extract (Table 3).

2.3.1.2 Hormone measurements

The LH level of the 2010 highest extract concentration was significantly decreased, however no statistical differences in the increase of E2, FSH or P4 quantization were registered (Figure 4). Estrone (E1) levels were not modified by the treatments. TSH levels were significantly

decreased in the highest dose too, but no clear changes in T3 and T4 levels were observed (Figure 5).

2.2.1.4 Gene transcripts

There was no clear changes in transcript levels of the target genes investigated in the uterus of female rats examined during the estrus.

3. Discussion

Water is that is more critical to the maintenance of life than food [23] and it is well established that sewage effluents are the main source of water contamination [1, 2]. The endocrine system has a finite capacity of homeostasis in face of constantly changing environment [24]. Then, concerns has been raised about possible harmful effects of chemicals, drugs, natural and synthetic hormones, and others on the endocrine system of humans and wildlife [25]. Studies carried out in Brazil confirmed that sewage discharges, with or without previous treatment, play an essential role on the occurrence of estrogens and xenoestrogens in waters [3, 26, 27]. Drinking water distributed to an specific city in São Paulo State (Campinas) was used in this study, but the same scenario is relevant at many places around the world.

The basic questions regarding this matter remains: are these chemicals in sufficient concentrations to induce endocrine impairment? What can be detected in surface water is relevant enough to care about? Trying to answer these questions, we started with chemical characterization of DW collected from a sampling site that has been extensively evaluated in earlier studies[4, 5, 7, 27]. The surface water that supplies the drinking water treatment plant where the samples were collected receives large amounts of treated and also raw sewage discharges. Then, the emerging contaminants levels in DW were higher than median values around the world[4] and presented besides classical hormones, caffeine [9] and some classes of pesticides. Although caffeine has been used as a tracer for sewage contamination some

studies demonstrated that it can cause adverse effects in endocrine tissues, changes in developmental parameters and in hormone levels in rodent assays [28-30]. In female rats, atrazine has also been shown to exert effects on several reproductive and endocrine parameters, including blunting of the hormone-induced LH surge and inhibition of pulsatile LH release [31].

As guided by many international protocols on standard regulation, the next logical steps to follow would be to use a battery of tests to analyze the potential endocrine disruption of these water samples. First, we conducted an *in vitro* receptor binding assay (BLYES/BLYAS) as the most relevant pathway of endocrine disruption (ED). Previous samples collected at the same site showed positive results in the BLYES assay[6]. Surprisingly the two DW samples analyzed in this study were negative for BLYES/BLYAS assay, but this is also expected considering the complexity of environmental matrices and variability on the mixture composition. Another explanation would be that the endocrine disruptors present in those two water samples, and confirmed by the chemical analysis, were in low levels and/or did not have enough potency to bind to the proper receptors. The intrinsic potency of an endocrine disruptor is dependent upon parameters of affinity and efficacy, as well as amount of receptor number and ligand bioavailability [24].

Although *in vitro* assays provide useful information on potential modes of action, previous studies have suggested that *in vivo* data should carry more weight when determining the need for other tests in a screening programs[32]. Then, we continued with Tier 1 *in vivo* assays, where we could explore more ED pathways and possible synergistic effects of low dose compounds after physiological metabolic pathways. We chose the uterotrophic assay, a very sensitive bioassay for estrogenic activity, using intact immature female rats that still have immature hypothalamic-pituitary-gonadal (HPG) axis. This assay covers a larger scope of investigation, because it can respond to substances that interact with the HPG axis rather than just the estrogen receptor [33]. After just 3 days of exposure to the 2010 DW extract, we observed a

tendency towards increased uterus weight, vaginal keratinization and significant increases of LH and FSH levels. Then, we tested another water sample collected in 2012 and developed a female pubertal assay, which has a longer time of exposure, using the 2010. Because atrazine was detected in the 2010 DW sample, we also included a control group with this pesticide administered alone and equivalent to the highest dose water extract amount (3.73 ng/l/kg bw). The same parameters were evaluated for this group and since data was similar to the negative control, it was assumed that the amount of atrazine found in the water samples tested did not exert any effect in the tested conditions. Another animal group was included to test if the extraction procedure could elicit some influence in the endocrine disrupting responses: animals received daily by gavage only ultrapure water for 3 days. All parameters did not differ from the group receiving ultrapure water extract eluted in 1% DMSO (data not shown). These results also excluded the possibility of DMSO interference [34].

We also observed that, after the 3-day treatment with the 2012 DW samples, the tendency towards increased uterus weight was repeated. Otherwise, no other response appeared to be the same as with the 2010 water sample in this assay. Is already known that the concentration of the natural hormone 17-β-estradiol is consistently low throughout the prepubertal development and increase after the PND 28th [35]; the gonadotropin and prolactin surges at the day of vaginal opening which resulted in a subnormal progesterone release. These levels are latter (between day 20 and puberty) decreased by an inhibitory action of prolactin and/or progesterone on pituitary gonadotropin release [36]. Some of this hormonal fluctuation was observed in our both studies, especially the serum LH levels. It is also recognized that disruption of GnRH/LH pulsatility leads to a reduction in gonadotrophin secretion and to a loss of reproductive function [37, 38]. Then, alteration of the HPG hormonal release during the prepupertal development could reflect in the LH secretion found in the adult age, like we found in our studies, and finally leads to estrous cycling deregulation. This possible HPG-treatment interaction could also explain the significant decreased levels of TSH in adult female

rats exposed to the high doses of the 2010 DW extract. However, our data could also represent a pulse of these hormone release cycle at the moment of euthanasia. A serial blood collection would be more appropriate to the evaluation of hormone levels, and then could better confirm a LH release disruption and all hormonal alterations registered. The possibility exists that with an extended period of treatment, we could also find thyroid follicular cells alterations, and recognize which organs are directly affected by this treatment. The reduction in body weight gain of these animals could also indicate the severity of chronic exposure to the contaminants and more time of treatment could induce other alterations that were not seen at the adopted observation time.

When comparing the results of the two female rats protocols, i.e., the uterothrophic and the prepubertal female assay, the immature HPG axis seemed to be more sensitive to the chemicals present in the water samples, and the results should not just be taken as flags of concern, because possibly this time-phase interaction could influence other stages of maturation. It is clear that we should prompt additional investigations to determine adverse effects in mammal's endocrine tissues under development. The evidence found in the rats cannot be considered as adverse effects nor endpoints *per se*, but are important preliminary findings of a potential DW endocrine disrupting activity that could be linked to some humans diseases, such as women sub-fertility and others[39] . It is also important to highlight that environmental samples chemical composition are underestimated, because of the technical procedures involved, and then our real exposition could be even higher based on a entire life consumption.

Mostly due to endocrine disruption studies came a reverse approach in toxicology, in which identification of pathogenesis mechanisms comes first followed by research to define possible consequences of activating these mechanisms [24]. Then, testing mixtures or real water samples for endocrine disrupting activity is challenging and early effects can be subtle, epigenetic and

or gene expression alterations [40, 41]. The high throughput evaluation using endocrine gene transcripts is a very sensitive baseline to endocrine activity, and combined with protein validation assays, provides a full screening of a substance mechanism of action. Our data highlights estrogen and progesterone receptors responses, which are responsible for remodeling the uterus growth[42], and also two endocrine target genes Hoxa-10 and Calbindin-D9k (CaBP-9k), which are regulated by progesterone and estrogen exposure, respectively [43, 44]. Hox genes are evolutionary conserved and necessary for body axis patterning during embriogenesis and adult functional differentiation[43]. Hox multigene family homeobox A, Hoxa-10, is affected by bisphenol A (BPA), methoxichlor and diethylstilbestrol (DES) after *in vivo* and *in vitro* exposures [44-46]. It has been reported that CaBP-9k (from a cytosolic calcium binding protein group) is rapidly and strongly induced in the uterus of mammals by environmental estrogenic compounds, like DES[47]. Like other results obtained in the present study, gene transcript evaluations also did not show significant expression levels difference after 17-α-ethynylestradiol exposure, but HOXa-10 and CaBP-9k showed a treatment variation after the 2010 and 2012 (not shown) water extract exposures: the expression levels of HOXa-10 were reduced and the CaBP-9k were increased. We also conducted imunohistochemistry of ER and PR in the uterus of these rats, but the results were negative or inconclusive (not shown).

Other study developed by our research group evaluated the potential for ED of sewage sludge samples collected from the same region in São Paulo State. Young adult male rats were exposed for 8 weeks to a sludge sample containing dioxins, furanes, PCBs and PAHs[48], and they found FSH levels alteration and sperm quality impairment[49].

Therefore the DW selected for this study may possess endocrine disruption activity and could alter female rats physiology. The significant resources dedicated to developing and validating the bioassays conducted in this study have led to an improved understanding of the challenges involved with implementing and interpreting endocrine disruptors assays. In this regard, we

suggest some major issues for research in the hazard identification of endocrine disruptors: a) when investigating environmental samples, its content could be variable, but the chemical characterization would help to delineate further experiments; b) use of more comprehensive *in vitro* batteries reaching different modes of action can help clarify *in vivo* assays results; c) improve *in vivo* assays including end-points for the entire system analysis, also induced in long-term effects upon exposure during susceptible developmental phases. Specifically in the *in vivo* assays, we could also include more sensitive and high throughput parameters of evaluation, such as hormone analyses in a serial blood collection, organs histology and morphometry, and if some evidence were found, imunohistochemistry of enzymes and receptors in target tissues, followed by transcripts expression of susceptible genes. Whenever possible, include additional positive controls for the suspected ED pathway as a reference, and in a dose comparable to the amount found in the sample.

As a suggestion to complement the ED evaluation first screening, other tests would be added to elucidate the mode of action and species/gender susceptibility, like the steroidogenesis - human cell line or aromatase - human recombinant microsomes, Hershberger or pubertal male assays, amphibian metamorphosis and fish short-term reproduction. In an overall, these suggestions mean more studies and end-points inclusion in endocrine disruption guidelines. The lessons learned will be also beneficial to eventual implementation of the yeast assay and the uterotropic and pubertal assays in a water quality analysis proposition for our country and maybe for others too.

4. Material and methods

4.1 Water samples collection and pre-concentration

Two DW samples were collected during the dry season of 2010 and 2012 from a tap water fountain at Campinas, São Paulo State, Brazil. That water fountain site is supplied by a Water Treatment Plant (WTP) which treats raw water from the Atibaia River, which has high levels of

domestic and industrial pollution. After conventional treatment followed by disinfection with chloroamine, the WTP provides DW for nearly 950.000 inhabitants at Campinas city, SP.

The samples were collected in previously cleaned amber glass bottles and forwarded to the laboratory, where they passed through glass fiber filters (Schleicher & Schuell, Dassel, Germany) and then filtered using 0.45 µm pore size cellulose acetate membranes (Sartorius, Geottingen, Germany). Solid-phase extraction of drinking (4 L) waters were carried out using 500 mg HLB Oasis cartridges (Waters, Milford, USA) fitted to a lab-made extraction system [50]. Cartridge conditioning and analyte elution were performed with methanol as described elsewhere [5]. Extracts were used for chemical and biochemical analyses, and were finally eluted in 1% DMSO for the in vivo assays.

4.2. Chemical analysis

Emerging contaminants were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS-ESI) (Agilent 1200 LC system coupled to an Agilent 6410 TripleQuad mass spectrometer) [5, 6]. Representative pesticides were selected based on a São Paulo State, Brazil priority pesticides list, which criterion was usage and occurrence in water destinated to human population[51]. They belonged to different pesticides types (acaricides, insecticides, fungicides, herbicides) from six different chemical groups: triazoles (difenoconazole, epoxiconazole, tebuconazole), triazines (atrazine), strobilurins (azoxystrobin, picoxystrobin, pyraclostrobin, trifloxystrobin), organophosphates (chlorpyrifos, profenofos), phenyl pyrazole (fipronil) and benzimidazole (carbendazim). Other compounds were also evaluated and were already detected elsewhere[3-7, 27, 52, 53]: caffeine, bisphenol A, natural and synthetic hormones (estrone, 17β-estradiol, estriol, progesterone, testosterone, mestranol, levonorgestrel, dietilstilbestrol, 17α-ethynylestradiol), phenols (phenolphthalein, 4-n-nonylphenol, 4-n-octylphenol), bactericide (triclosan), inseticides (imidacloprid, hexythiazox), and herbicide (bromacil). Most of these substances do not have established DW standards or health

advisories at this time. Table 1 shows the Limits of Quantification (LOQ) for the targeted chemical compounds.

4.4 *In vivo* assessment

4.4.1 Animals and housing

Studies were performed after approval of the Ethical Committee on Animal Experimentation (Protocol CEEA 823-2010, Botucatu Medical School, UNESP). Newborn female *Wistar* rats (Multidisciplinary Center for Biological Investigation - CEMIB, Brazil) were weaned, weighed and allocated to treatment groups at postnatal day (PND) 21. The animals were maintained in an environmentally controlled facility: room temperature at 22 ± 2°C, humidity at 55 ± 10%, and 12-h light/dark cycle. Rats were allocated 3 animals per propylene cage with autoclaved pinewood bedding changed three times a week. They were fed a balanced laboratory diet (pellet chow Purina-Labina Evialis, Paulínia, SP) with genistein-equivalent content under the limits of the Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Female Rats guideline [54]. Ultrapure water was provided *ad libitum* through glass bottles renovated three times a week since the animals were born.

4.4.2 Dose selection

Experimental design is presented in Figure 1. Female rats were exposed by daily gavage to DW extracts during 3 (uterotrophic bioassay) [33] or 20 days (pubertal development female rat assay) [54] at 33.3, 166.5, and 333.0 mL equivalent of water/kg body weight, modeling a daily ingestion of 2 L, 5 L and 10 L of DW by a 60 kg human being [55]. In both studies, negative control group also was exposed to a ultra-pure water extract at the highest dose (333.0 mL/kg bw) of the DW extract samples. The extracts were daily administered in every morning using 1 ml ultrapure water/kg bw as vehicle.

4.4.3 Uterotropic bioassay

This is one of the most widely used assays to detect estrogenic substances[56]. The immature model, which has an intact HPG axis, can detect aromatizable androgens and is more sensitive to body weight-mediated changes in the uterus weights[24]. Female rats were weighed, dosed and checked for vaginal opening (VO) daily from PND 22 to 25. The groups exposed to the water extracts consisted of 12 animals each; an additional group was administered daily via gavage 17-α-ethynylestradiol (1 µg/kg bw) eluted in corn oil during 3 days as a positive control for estrogenicity. All females were sacrificed in the morning 24 hours after the last dose by exsanguination under deep anesthesia in a CO_2 chamber. Blood samples from the left ventricle were collected from each animal. Serum samples were prepared and stored at -25°C until analyzed for sex hormone concentrations. At necropsy, the reproductive organs were excised from each rat, trimmed free of fat and connective tissues, and weighed. Wet and blotted uterus were weighed. The wet weight includes the uterus and the luminal fluid content. The blotted weight was measured after the luminal content of the uterus had been expelled and removed. The pituitary and the thyroid glands were also excised and weighed after formalin fixation. Uterus samples of six animals from each group were flash-frozen in liquid nitrogen and stored at -80°C until transcriptomic analyses.

4.4.4 Pubertal development and thyroid function in intact juvenile/peripubertal female rats

The pubertal assay is capable of detecting EDs that operate at a variety of modes of action: strong and weak (anti)estrogenic and (anti)androgenic compounds, steroid biosynthesis, aromatase or 5α-reductase inhibitors, thyroid-active agents and compounds affecting the HPG or HPT axis[24]. This pubertal development assay was conducted only with the DW sample collected in 2010. Fifteen animals per dose group selection were weighed, dosed and checked for VO daily from PND 22 to 42. This duration of treatment is unnecessary to detect estrogenic chemicals, but is required for the detection of pubertal delay and anti-thyroid effects [54]. Beginning on the day of VO, through to and including the day of necropsy, daily vaginal smears

397 were obtained and evaluated under a low-power light microscope for the presence of
398 leucocytes, nucleated epithelial cells, or cornified epithelial cells [57]. Systemic serum and organs
399 collection and procedures were the same as for the uterotrophic assay.

400 **4.5 Hormone measurements**

401 Serum samples were assayed for follicle-stimulating hormone (FSH) and luteinizing hormone
402 (LH) by double-antibody radioimmunoassay (RIA) with specific kits provided by the National
403 Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (NIADDK, Baltimore, MD, USA).
404 The FSH primary antibody was anti-rat FSH-S11, and the standard was FSH-RP2. The antiserum
405 for LH was LH-S10 using RP3 as reference. The lower limit of detection for FSH and LH was 0.2
406 ng/mL and the intra-assay coefficients of variation were 3% and 4%, respectively. Serum
407 concentrations of E2, T and P4 were determined using Estradiol and Testosterone MP
408 Biomedicals (Solon, Ohio, USA), and Progesterone Maia kits (Biochem Immunosystems,
409 Serotec, Italy), respectively. The intra-assay coefficient of variation were, respectively, 2.5% for
410 E2 and 3.7% for P4. Estrone was also measured using DSL-8700 kit (Webstern, Texas, USA),
411 with an intra-assay error of 3.5%, and Prolactin (PRL) with MP Biomedical kit (Santa Ana, Ca,
412 USA), - error of 3.4%. The thyroid-stimulating hormone (TSH), total triiodothyronine (T3) and
413 thyroxine (T4) were determined using Coat-a-Count Siemens kits (Munich, Germain), and the
414 errors were 2.8-4% .

415 In the pubertal development/thyroid function in intact juvenile/peripubertal female rat assay,
416 only female rats in estrus cycle at the moment of euthanasia were analyzed for steroid
417 hormone levels. In order to prevent inter-assay variation, all samples were assayed in the same
418 RIA.

419 **4.6 Analysis of reproductive organs**

At necropsy, ovaries, adrenal glands, uterus, vagina, pituitary and the thyroid glands, liver, were excised from each rat, fixed in 10% buffered formalin and processed for histological analysis using hematoxylin and eosin (H&E) staining. Histology evaluation was according to the Guidance Document For Histologic Evaluation Of Endocrine And Reproductive Tests In Rodents [58]. The vaginal cornification assay for detecting histological changes in vaginal epithelium is widely used although it is largely qualitative[25].

From each ovary, three 4 µm thick sections per animal, 40 µm apart, were obtained, mounted on glass slides and stained with H&E. Ovarian follicles and corpora lutea from the right ovaries were quantified in 3 sections per animal [59]. Follicles were classified according to Guideline OECD 2009 (11) - Part 3 [58]. An optical microscope (40X) coupled to a digital camera and a PC with software Image-Pro Plus (Media Cybernetics, MD- USA) was used. Image analyses and tissue area measurements were executed with the software Image-J, available at http://rsbweb.nih.gov/ij/. This evaluation was only conducted in the rats exposed to the 2010 water sample.

In the uterus, the endometrial thickness was measured in 3 sections per animal, using a light microscope. In each section, five different regions were analyzed, resulting in a total of 15 measurements per animal [59]. The same image system used in the ovaries was utilized to measure the endometrial thickness.

4.8 Gene transcripts measurements

4.8.1 Total RNA isolation

Total RNA was isolated from uterus of 5 animals per group using RNeasy Mini kit -Qiazol (Qiagen, 74804) in accordance with the manufacturer's instructions. The resulting total RNA was quantified using Nanodrop ND-1000 (NanoDrop Technologies, Inc.). RNA integrity (28S/18S ratio) and purity was assessed using RNA 6000 nano assay LabChips® (Agilent

Technologies, 5067-1513) and analyzed on a 2100 Bioanalyzer (Agilent Technologies). Only samples with an RNA integrity number (RIN) > 7.0 (Agilent software) were employed for further analyses.

4.8.2 RT-qPCR analysis

A real-time quantitative reverse-transcriptase-polymerase chain reaction (RT-qPCR) approach was used with selected transcripts and with Applied Biosystems reagents and equipments. cDNA was synthesized with 2ug of total uterus RNA from control and treated samples, through a reverse transcriptions with random hexamer primer using the High Capacity cDNA Reverse Transcription kits (4374966) in accordance with the manufacturer's protocol. RT-qPCR reactions were performed using Taqman probes (Assay on demand - 4331182, 4331372; RefSeq) and Taqman Universal PCR Master Mix (4364340) on a Step One Plus machine. The genes studied were: Er-α (NM_012689.1, 87pb), Er-β (NM_012754.1, 89pb), Pgr (NM_022847.1, 105pb), Cabp (NM_012521.1, 99pb) and Hoxa 10 (NM_001129878.1, 90pb).

4.9 Statistical analyses

Statistical analyses were performed using Jandel Sigma Stat software (Jandel Corporation, San Rafael, CA). Values of final body weights and body weight gain, relative and absolute organ weights, food consumption, and hormone data were analyzed by analysis of variance (ANOVA), and data were subjected to the Kruskal–Wallis followed by the Dunn's test to determine significance. The number of animals with vaginal keratinization in the uterotropic assay and all the pubertal parameters of the peripubertal development bioassay were analyzed by Qui-square test, follow by the Fischer test when necessary. The criterion for significance was set at $p < 0.05$, compared to the group treated with ultrapure water extract.

4.3 Bioluminescent assay

A *Saccharomyces cerevisiae* bioluminescent bioreporter assay (Bioluminescent yeast estrogen/androgen human receptor assay - BLYES/BLYAS) was used to evaluate the estrogenic and androgenic activity of the water samples, respectively. This specific bioluminescent strain was described by Sanseverino et al. [60]. The protocols follow studies described elsewhere [6, 61, 62].

5. Conflict of interest

None.

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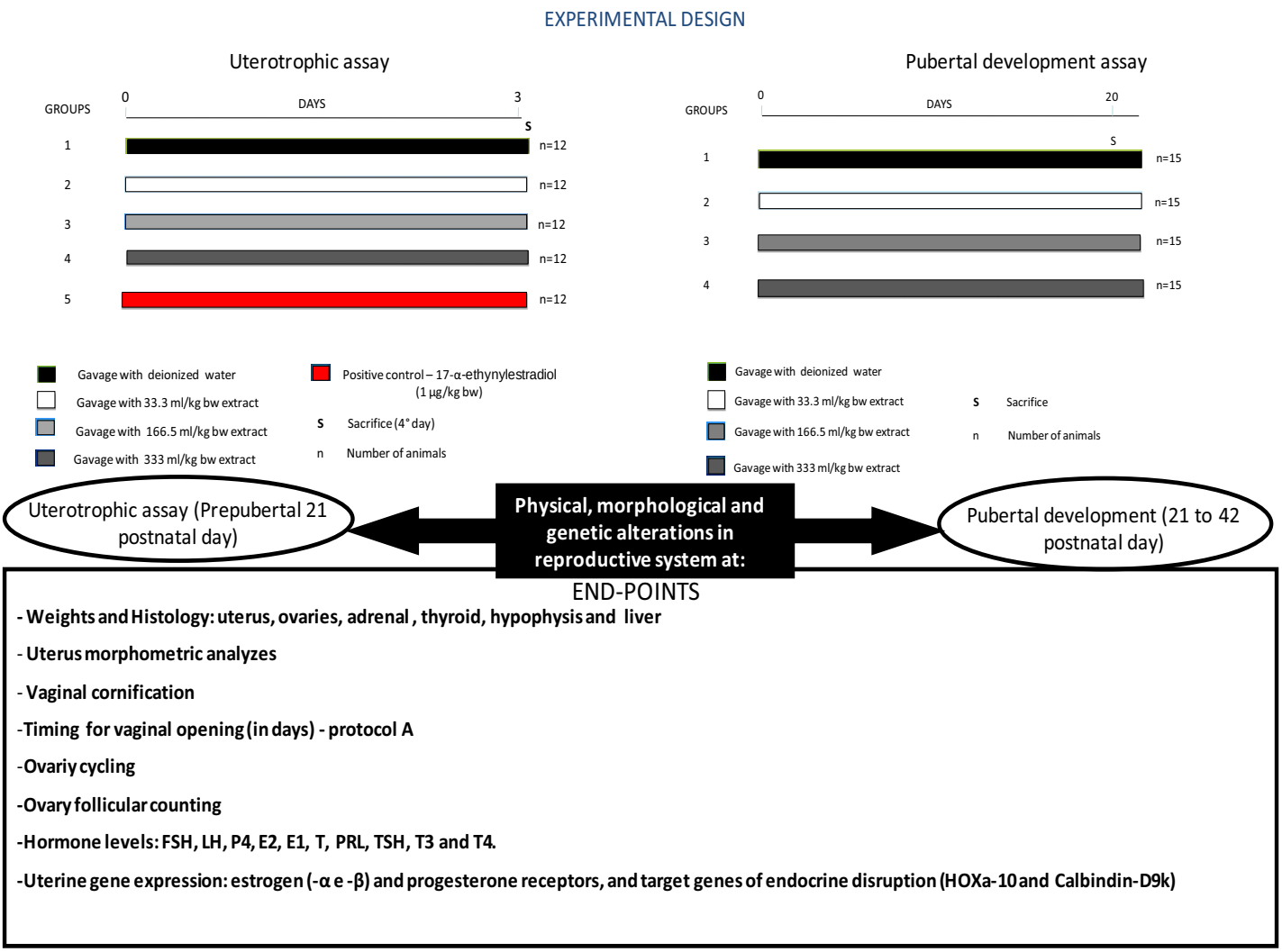

Figure 1. *In vivo* experimental design of the drinking water extracts.

Table 1. Chemical analyses indicated as Limit of Quantification (LOQ) levels and estrogen/androgen activity of target compounds evaluated in the 2010/2012 drinking water extracts.

Target compounds	Compounds identified by LC-MS/MS-ESI* analyses		BLYES/BLYAS Estrogen/Androgen activity *in vitro* (ng E2/T equivalent/L)
	2010 water sample	*2012 water sample*	*2010 or 2012 water samples*
Atrazine (ATZ)	**11.2ng/L**	**0.81 ng/L**	Not detected
Caffeine (CAF)	**5.8 ug/L**	**0.056 ug/L**	
Estrone (E1)	**1 ng/L**	< 2.6 ng/L	
Carbendazim (CBZ)	Not analysed	**0.22 ng/L**	
Imidacloprid		**0.88 ng/L**	
Azoxistrobina (A26)		**0.23 ng/L**	
Tebuconazole (188)		**0.19 ng/L**	
Bisphenol A (BPA)	< 1.5 ng/L	< 1.5 ng/L	
Dietilstilbestrol (DES)	< 3.4 ng/L	< 3.4 ng/L	
17β-estradiol (E2)	< 5.9 ng/L	< 5.9 ng/L	
Estriol (E3)	< 0.8 ng/L	< 0.8 ng/L	
17α-ethynylestradiol (EE2)	< 13.9 ng/L	< 13.9 ng/L	
Phenolphthalein(PhPh)	< 3.7 ng/L	< 3.7 ng/L	
4-n-nonylphenol (nNP)	< 10.6 ng/L	< 10.6 ng/L	
4-n-octylphenol (nOP)	< 5.1 ng/L	< 5.1 ng/L	
Mestranol (MEE)	< 8.8 ng/L	< 8.8 ng/L	
Levonorgestrel (NGT)	< 0.9 ng/L	< 0.9 ng/L	
Progesterone (P4)	< 2.7 ng/L	< 2.7 ng/L	
Testosterone (T)	< 1.7 ng/L	< 1.7 ng/L	
Triclosan (TCS)	< 0.7 ng/L	< 0.7 ng/L	
Difenoconazole	Not analysed	<0.2 ng/L	
Epoxiconazole		<0.7 ng/L	
Azoxystrobin		<0.2 ng/L	
Pyraclostrobin		<0.3 ng/L	
Picoxystrobin		<0.7 ng/L	
Trifloxystrobin		<0.2 ng/L	
Chlorpyrifos		<0.9 ng/L	
Profenofos		<1.5 ng/L	
Fipronil		<18 ng/L	
Hexythiazox		<1.5 ng/L	
Bromacil		<0.5 ng/L	

* liquid chromatography tandem mass spectrometry

Table 2. Uterotrophic Assay: Absolute (g) and relative (%) blotted uterine weights[1], and endometrial thickness.

GROUPS	2010 sample		2012 sample		Endometrial thickness	
	Absolute weights	Relative weights	Absolute weights	Relative weights	2010 sample	2012 sample
Extract of ultrapure water	0.036±0.005	0.057±0.009	0.028±0.008	0.052±0.012	101.01±46.45	98.194± 43.517
Extract of water sample:33.3 ml/kg bw	0.040±0.008	0.067±0.016	0.032±0.015	0.058±0.032	109.55±48.78	107.308± 54.621
Extract of water sample:166.5 ml/kg bw	**0.048±0.011***	0.075±0.018	0.043±0.044	0.065±0.027	**154.15±54.28***	126.435±62.494
Extract of water sample:333 ml/kg bw	**0.046±0.014***	0.085±0.025	0.041±0.036	0.063±0.055	98.13±36.65	122.910±54.519
Positive control - 17α-ethynylestradiol(1ug/kg bw)	**0.122±0.016***	**0.215±0.028***	**0.149±0.046***	**0.279±0.073***	**216.93±57.47***	**185.099±85.488***

1 Mean ± SD and nonparametric; Means different from Extract of ultrapure water at *p<0.05.

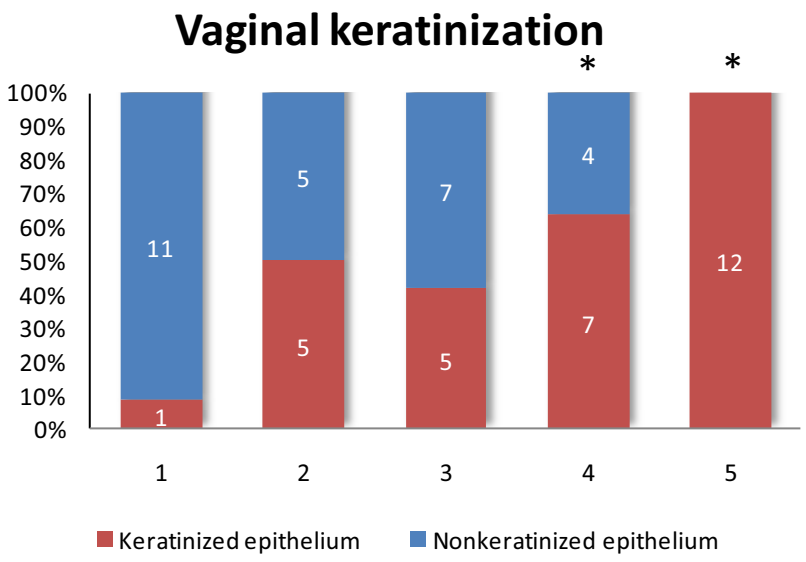

Figure 2. Number of animals with keratinization of the vaginal epithelium after 3 days of exposure to the 2010 drinking water extract. Groups: 1: ultrapure water; 2: 33.3 ml/kg bw extract; 3: 166.5 ml/kg bw extract; 4: 333 ml/kg bw extract; 5:17-α-ethynylestradiol (1 µg/kg bw). Different from Extract of ultrapure water at *p<0.05.

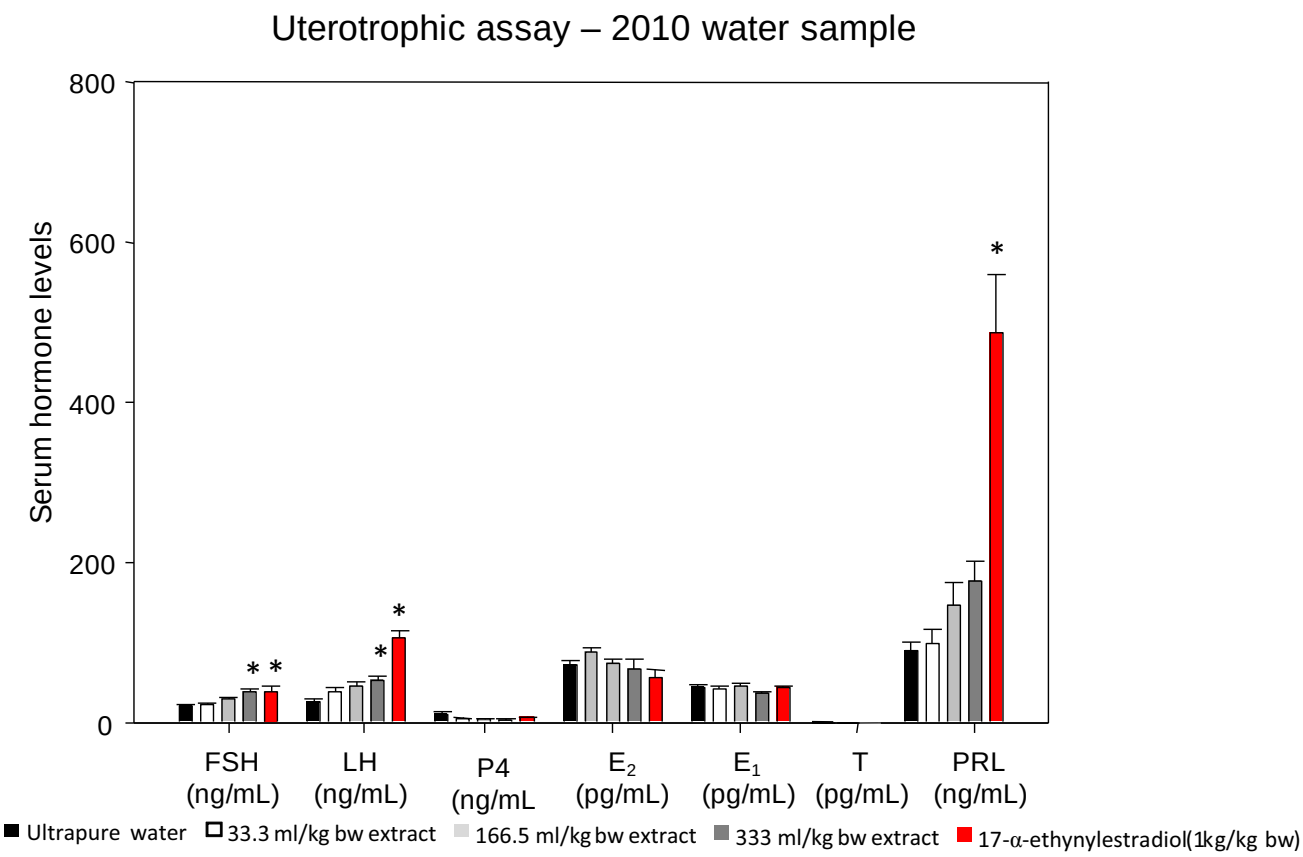

Figure 3. Hormone levels of immature rats exposed to 2010 water sample. FSH: follicle-stimulating hormone; LH: luteinizing hormone; P4: progesterone; E2: estradiol; E1: estrone; T: testosterone; PRL: prolactin. Different from Extract of ultrapure water at *p<0.05.

Table 3. Female pubertal parameters after 20 days of exposure to the 2010 drinking water extract.

Groups	Age at vaginal opening (days)[1]	Body weight at vaginal opening (g)[1]	Mean age at first vaginal estrous (days)	Mean cycle length (days)	Cycling (%)	Regularly cycling (%)
Extract of ultrapure water	36.47±1.41	125.07±12.8	35.8	4.9	100	93.3
Extract of water sample:33.3 ml/kg bw	35.40±2.13	124.53±12.23	36.4	5.4	100	**53.3***
Extract of water sample:166.5 ml/kg bw	35.27±1.75	121.0±9.39	35.8	5.2	100	66.7
Extract of water sample:333 ml/kg bw	35.87±2.36	123.73±12.7	36.7	5.3	93.3	80

[1]Values are mean ± SD. Nonparametric data; Different from Extract of ultrapure water at *p<0.05.

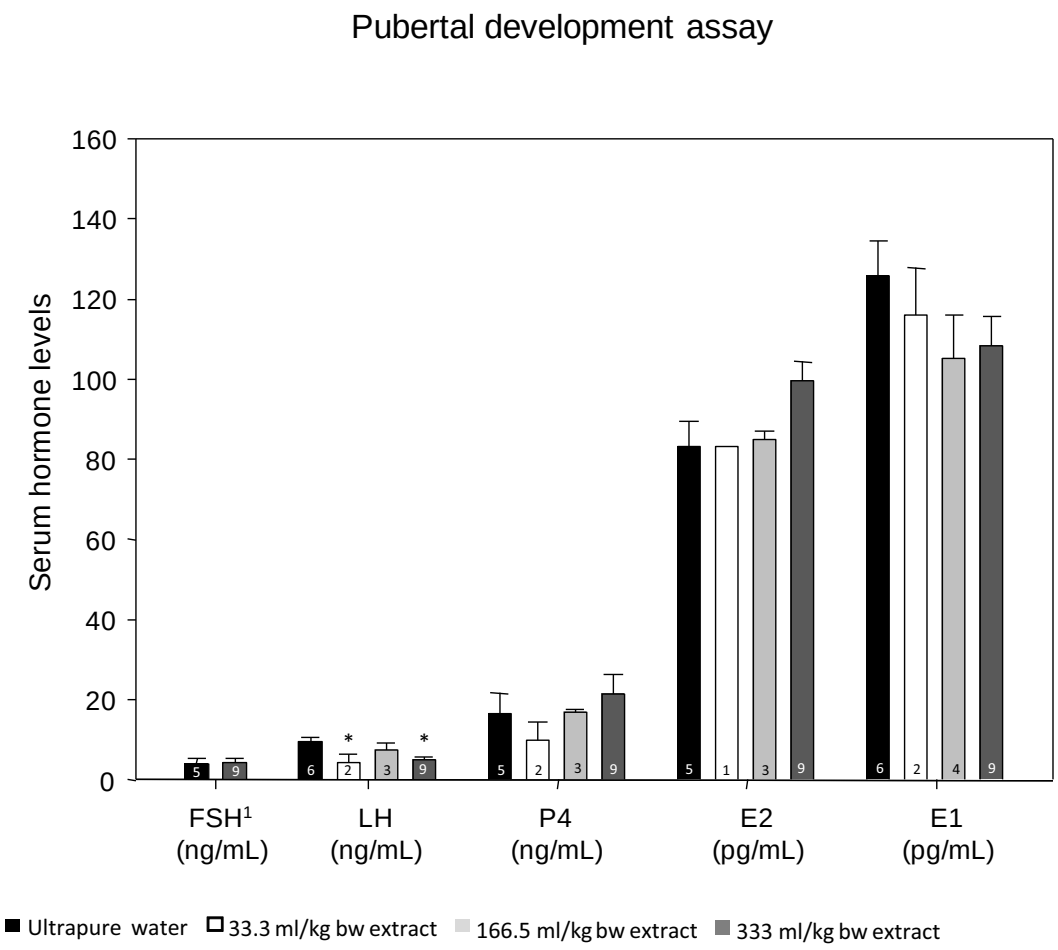

Figure 4. Hormone levels of female pubertal development assay exposed to 2010 water sample. Only animals in estrous cycle were analyzed (number of animals inside de columns). [1]: Not enough samples to analyze this hormone in the other treated groups. FSH: follicle-stimulating hormone; LH: luteinizing hormone; P4: progesterone; E2: estradiol; E1: estrone. Different from Extract of ultrapure water at *$p<0.05$.

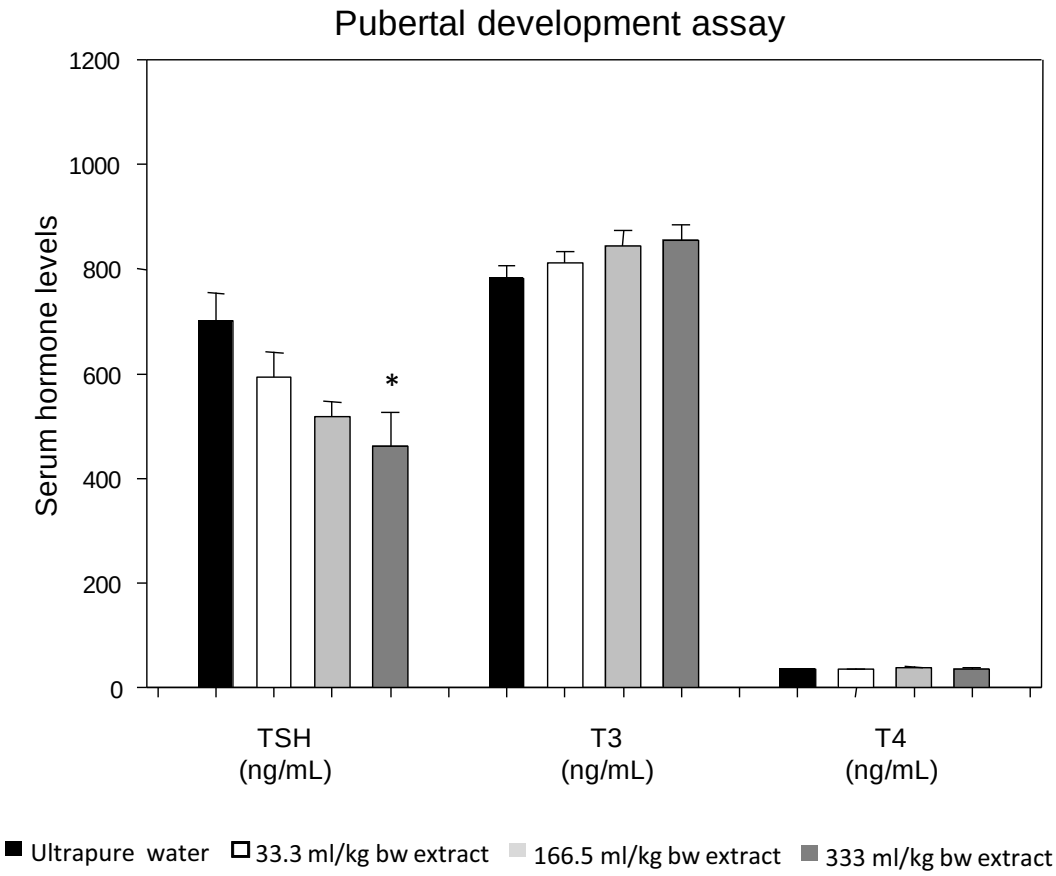

Figure 5. Thyroid hormone levels of female pubertal development assay exposed to 2010 water sample. TSH: thyroid-stimulating hormone; T3:triiodothyronine; T4:thyroxine. Different from the ultrapure water extract at *p<0.05.

Supplementary data

Table 1. Female Pubertal Assay: Body weight gain (g)[1], and absolute (g) and relative (%) blot uterine and liver weights[1]

	Body weight (g)[1]			Blotted uterus		Liver	
Groups	**Initial[2]**	**Final**	**Gain[3]**	**Absolute weight[2]**	**Relative weight**	**Absolute weight**	**Relative Weight[2]**
Negative control	40.07±5.86	154.67±10.04	114.6±6.66	0.24±0.07	0.37±0.10	7.39±0.52	4.79±0.341
Extract:33,3 ml/kg bw.	46.8±7.43	153.67±16.47	106.87±10.81	0.18±0.08	0.29±0.15	6.81±1.05	**4.42±0.36***
Extract:166,5 ml/kg bw.	44.27±4.57	147.8±9.54	**103.53±7.73***	0.20±0.08	0.30±0.13	**6.37±0.86***	**4.3±0.39***
Extract:333 ml/kg bw.	43.47±4.66	149.13±12.47	**105.67±9.26***	0.22±0.06	0.34±0.10	6.94±0.81	4.58±0.24

[1] Values are mean ± SD; [2] Nonparametric data; [3] Body weight gain: final minus initial body weight; Means different from Negative control at *p<0,05.

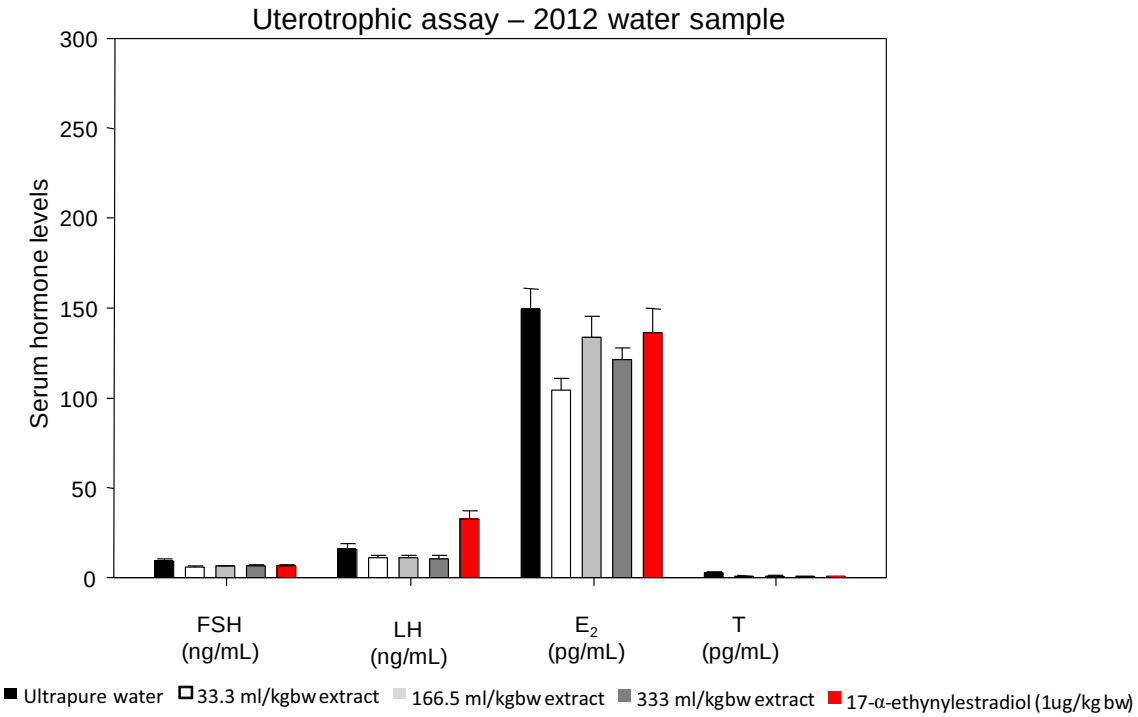

Figure 1. Hormone levels of immature rats exposed to 2012 water sample. FSH: follicle-stimulating hormone; LH: luteinizing hormone; E2: estradiol; T: testosterone. The values of T were multiplied by 10 to appear in the graphic. Different from the ultrapure water extract at *p<0.05.

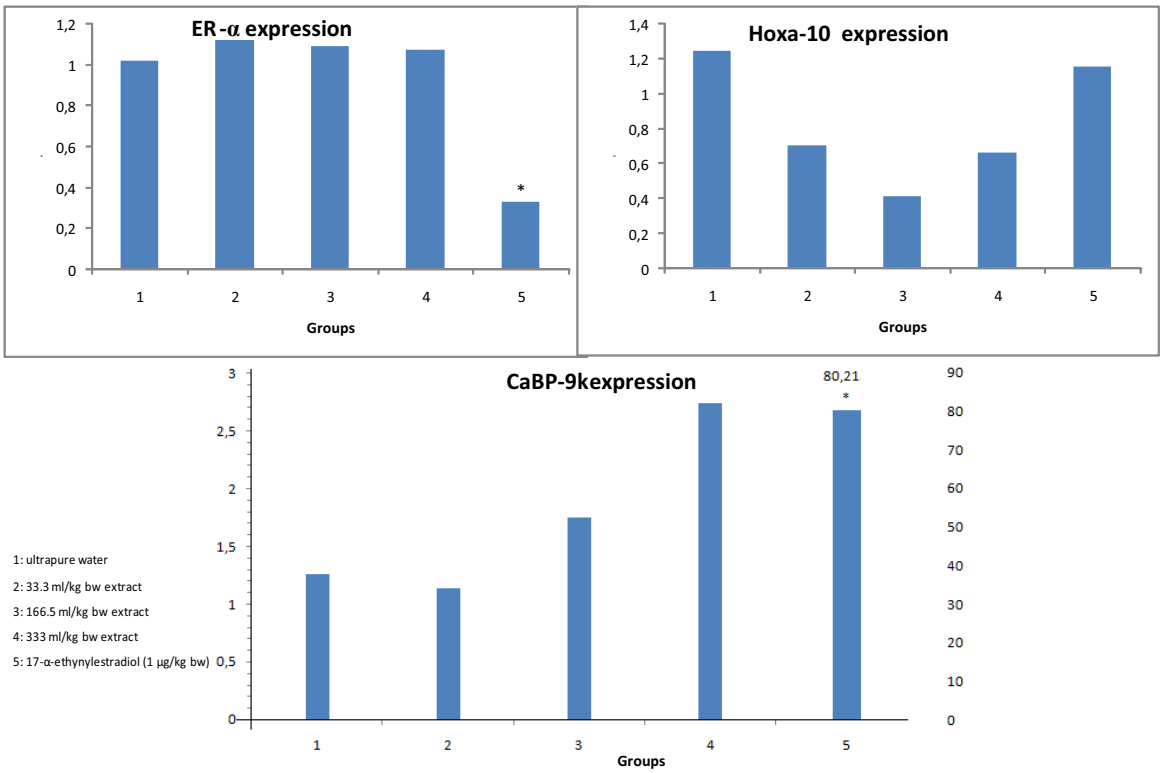

Figure 2. Relative quantization of target genes from immature female uterus rats after 3-days exposure to 2010 water sample.

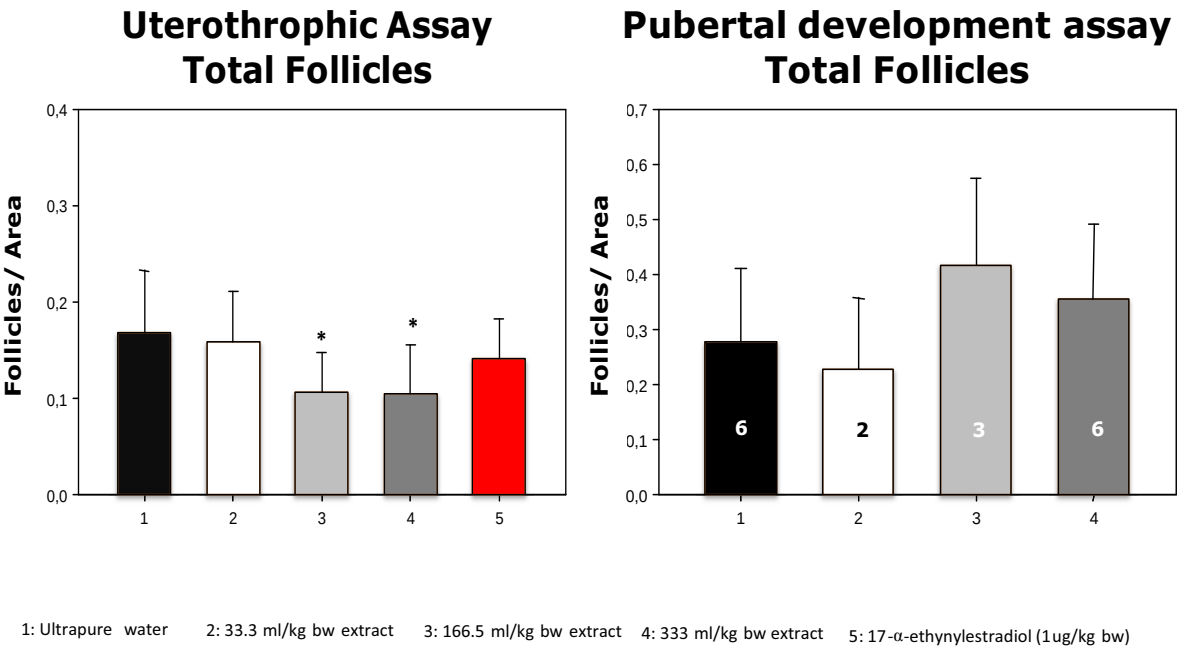

Figure 3. Number of total follicles per area in both studies with 2010 water sample. In the pubertal development study, only animals in estrous cycle were analyzed (number of animals inside de columns). Different from Extract of ultrapure water at *p<0.05.