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RESEARCH ARTICLE

Effects of waterborne luteinizing hormone and follicle-stimulating hormone on reproduction of the rotifer *Brachionus calyciflorus* (Monogononta: Brachionidae)

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Abstract – This study used freshwater rotifers to evaluate the effects of two endocrine disrupting compounds (EDCs), luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which can be considered emergent contaminants in urban and rural wastewater and are of ecotoxicological importance. LH stimulates the synthesis of testosterone, whereas FSH promotes the maturation of follicles and sperm in vertebrates and invertebrates. However, in rotifers, there are no reports of the effects of chronic exposure to these hormones when added to reconstituted culture medium, as a way to study potential adverse effects that might occur in the environment. Therefore, we studied the reproductive effects of the rotifer *Brachionus calyciflorus* Pallas 1766 using a 4-day reproductive assay. Our results indicate that LH has a significant effect in increasing the production of females, males, and cysts, while FSH had no significant effect compared to control treatment. Additionally, our results indicate that LH exposure resulted in 0.33% of organisms being deformed, whereas FSH exposure resulted in 1.09% of organisms being deformed. Deformations included: (a) abnormal growth of lorica, (b) joined foot-head, (c) deformed anterior spine, and (d) deformed parthenogenetic eggs. The organisms with LH-induced deformations did not reproduce and only lived 48 h after 4 days of exposure, while those with FSH-induced deformities survived 15 days and produced 105 cysts with a hatching percentage of 58.10%. Our goal was to contribute to the knowledge of endocrine systems and endocrine hormones of rotifers, to explain the potential mechanism of endocrine disruption that results in adverse effects in freshwater rotifers.

Keywords: Sexual reproduction / endocrine disruption / male inductions / abnormal rotifers / transgenerational effects

1 Introduction

The impact of endocrine disrupting compounds (EDCs) on aquatic biota is complex, because aquatic pollution is particularly troublesome to aquatic biota, which exhibit a continual life cycle and could have multigenerational exposure, resulting in the effects accumulating so slowly that major change goes undetected (Daughton and Ternes, 1999). EDCs released into freshwater bodies and the marine ecosystem is a serious ecological hazard, particularly because they are bioactive at low, environmentally relevant concentrations and possess a non-monotonic dose-response curve (Ciccotelli *et al.*, 1998; Grande *et al.*, 2007; Wojnarowics *et al.*, 2014).

For example, and relevant to our study, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) have been used for more than 30 yr for therapeutic ovarian stimulation for fertility treatments, and in the treatment of hypogonadotropic hypogonadism (Kenigsberg *et al.*, 1984; Bachmann *et al.*, 2015). That is, LH and FHS are widely used on infertility treatments to increase the number of oocytes (Santi *et al.*, 2017). Today, the brands Bravelle®, Gonal-F®, Puregon®, Elonva®, and Luveris® are commercially available; it is recommended, from day 3 to 9 of the menstrual cycle, that doses of up to 100 IU of FSH might be injected daily for timed intercourse or intrauterine inseminations, and doses of up to 300 IU for an IVF/ICSI medical procedure (Bachmann *et al.*, 2015). For example, in the brand Puregon®, which contains

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900 UI/1.08 ml, there is 83 μg/ml of FSH. Therefore, commercial infertility treatment products can be considered a potential pollution risk, considering the increase in infertility treatments and the scarcity of regulations. In fact, EDCs persist in water and other environments, and have been detected at concentrations on the order of nanograms or micrograms per liter in water (Wojnarowicz *et al.*, 2014; Vilela *et al.*, 2018). The presence of EDCs (including sexual hormones) in wastewater is caused for great concern because traditional water treatment does not efficiently remove many of these compounds (Bolong *et al.*, 2009).

Both FSH and LH are glycoprotein and gonadotrophic hormones that are pivotal endocrine regulators in sexual reproduction; the genes encoding predecessors of their subunits are found in most vertebrate and invertebrate species, although their roles are still unknown (Cahoreau *et al.*, 2015). In males, LH and FSH induce the synthesis of testosterone, but in females FSH triggers follicle maturation, and a sudden surge in LH drives ovulation (Roper *et al.*, 2015). However, in rotifers there are no reports of the effects of chronic exposure to these hormones when added to reconstituted culture medium, as a way to study potential adverse effects that might occur in the environment.

Sexual reproduction in rotifers depends on external and internal stimuli such as: (a) temperature, (b) photoperiod, (c) food quality, and, more importantly, (d) overcrowding, and (e) neurotransmitters, which are hormones that can trigger mechanisms like signaling and cellular communication in rotifers (Gallardo *et al.*, 1997; Gallardo *et al.*, 1999; Gallardo *et al.*, 2000; Pérez-Legaspi *et al.*, 2008; Alvarado-Flores *et al.*, 2009; Stout *et al.*, 2010; Yang and Snell, 2010). Consequently, to the best of our knowledge, pulsatile secretion of a similar hormone to gonadotropin-releasing hormone (GnRH) by the cerebral ganglia of rotifers in response to multiple stimuli recognized by mechanoreceptors and chemoreceptors stimulates the secretion of LH and FSH, both previously detected in the rotifer *Brachionus calyciflorus*, and LH has been detected in the head region of the male (Alvarado-Flores *et al.*, 2009). FSH mainly enhances the parthenogenetic cycle (mitosis), and results in increased production of estrogens that stimulate the release of large amounts of LH; this causes an increase in the production of amphoteric females capable of producing large amounts of haploid eggs that develop into males. This suggests that LH in this sexual stage of the rotifer life cycle enhances the increase of males and intensifies the probability of copula and cyst production.

Therefore, the effects of LH and FSH on the rotifer *B. calyciflorus* were analyzed following their addition to culture media, and studied using a 4-day chronic test. Because it is important to elucidate the adverse effects on aquatic biota as well as water quality, we used rotifers as a bio-indicator to determine the potential hazard of EDCs, considering their taxonomic group as a good indicator of a healthy ecosystem (Rico-Martinez *et al.*, 2017). We quantified the following indices: (a) population growth rate, (b) total production of sexual and asexual eggs, (c) production of cysts, (d) percentage of cyst hatching, (e) production of males, and (f) percentages of morphological abnormalities.

2 Materials and methods

The freshwater rotifer *B. calyciflorus* (originally collected in Gainesville, Florida, USA) was cultured in a bioclimatic chamber (Revco Co., USA), with a 16:8 dark–light period, with fluorescent lamps (600 × 1100 lux), and a temperature of 25 ± 2 °C according to Pérez-Legaspi and Rico-Martínez (1998). Rotifers were kept in Petri dishes containing EPA medium as described by the United States Environment Protection Agency (US EPA, 1985), this medium contains $NaHCO_3$ 96 mg/L, $CaSO_4 \cdot 2H_2O$ 60 mg/L, $MgSO_4 \cdot 7H_2O$ 60 mg/L, and KCl 4 mg/L; at pH 7.5. In addition, rotifers were fed with the green alga *Nannochloropsis oculata* (strain LB2164 of the University of Texas Collection) grown in Bolds Basal Medium as described by Nichols (1973). The *N. oculata* cultures were also maintained in the bioclimatic chamber (Revco Co., USA). Typically, 10–15 Petri dishes, each containing from 100 to 500 rotifer females, were cultured.

The effects of LH and FSH hormones (both acquired from Sigma-Aldrich) were screened in *B. calyciflorus* using a 4-day reproduction test. Five different nominal concentrations were selected, according to the exposure range to be explored, as well as based on published doses in previous publications involving oocyte and embryo experiments (Raju *et al.*, 2013; Bachmann *et al.*, 2015). We placed five neonates (<24 h old) in 2 ml EPA medium (negative control) with 5×10^6 cells/ml of the green alga *N. oculata*. The algal cells were counted using a hemocytometer (Neubauer, Marienfeld). This protocol represents a slight modification from that of Snell and Moffat (1992), because we used 24-well polystyrene plates (Costar Co., USA) instead of test tubes. The test conditions were the same in both protocols: 25 °C, pH 7.4–7.8, hardness 80–100 mg/L CaCO. Six replicates were performed for the control and each concentration; the concentrations of LH and FSH used were: 0.05, 0.1, 0.2, 0.3, 0.4, and 0.7 mg/L (nominal concentrations). At the end of the test, all rotifers and eggs were placed into a glass Petri dish, and the number of non-ovigerous females, ovigerous asexual females, ovigerous unfertilized sexual females, ovigerous fertilized sexual females, males, parthenogenetic (asexual or amictic) eggs, unfertilized sexual eggs, and fertilized sexual eggs (cysts) were counted. The population growth rate (r) of females, males, and eggs were expressed according to the formula:

$$r = \frac{\ln[Nt - No]}{T}$$

where $\ln(No)$ is the natural logarithm of the number of females in the plate after 4 days (T) and $\ln(Nt)$ is the natural logarithm of the initial number of rotifers in each plate (using only the N for females because the number of initial organisms was five).

For nonovigerous females, ovigerous asexual females, ovigerous unfertilized sexual females, ovigerous fertilized sexual females, male, parthenogenetic (asexual or amictic) eggs, unfertilized sexual eggs, and fertilized sexual eggs (resting eggs), No = zero. Ovigerous females were classified by the morphology of their eggs, which differed between females, males, and resting eggs (Wallace *et al.*, 2006). Abnormal (and normal rotifers) were

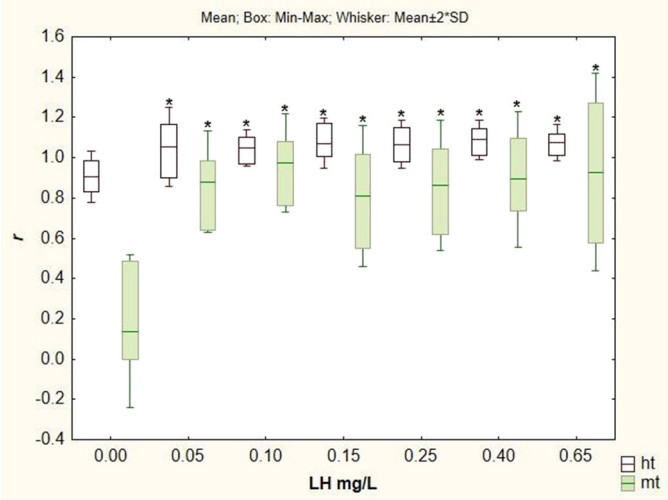

Fig. 1. Effects of LH on population growth rate of females (ht) and males (mt) of the rotifer *Brachionus calyciflorus*. Population growth rate (r) is offspring per female per day, and that of males depends on the production of the sex cycle of the female. Boxes indicate minimum and maximum values. Vertical lines on boxes indicate standard deviations. Asterisks (*) represent significant differences with respect to control ($p > 0.05$). $N = 6$.

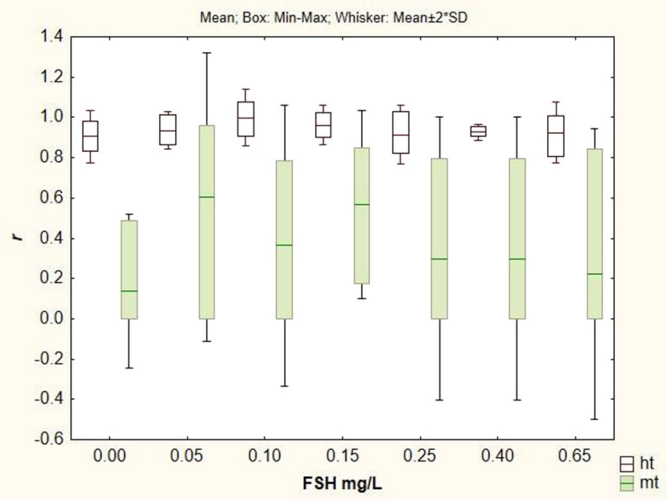

Fig. 2. Effects of FSH on population growth rate of females (ht) and males (mt) of the rotifer *Brachionus calyciflorus*. Population growth rate (r) is offspring per female per day, and that of males depends on the production of sex cycle of the female. Boxes indicate minimum and maximum values. Vertical lines on boxes indicate standard deviations. Asterisks (*) represent significant differences with respect to control ($p > 0.05$). $N = 6$.

observed at 20× magnification using a Leica DMLS inverted microscope and quantified.

The cysts collected from the 0.4 mg/L FSH exposure experiment were washed six times with EPA medium to eliminate any traces of FSH from the cyst surface. Then the cysts were incubated for 10 days at 4 °C in darkness to inhibit hatching. After the incubation period, 10 cysts were placed in 1 ml EPA medium in individual wells of 24-well polystyrene plates. The plates were placed in a bioclimatic chamber at 25 ± 2 °C and 16:8 light–darkness photoperiod to induce hatching. Finally, after 24 h, the number of hatched cysts was counted and compared with the number of cysts produced under normal culture conditions. We also evaluated the population growth rate (r) of the rotifers hatched from this experiment (under both control and FSH exposure conditions).

Using Statistica 7.0 software (StatSoft Inc., 2004), a one-way ANOVA was performed on the number of nonovigerous females, ovigerous asexual females, ovigerous unfertilized sexual females, ovigerous fertilized sexual females, males, parthenogenetic (asexual or amictic) eggs, unfertilized sexual eggs, and fertilized sexual eggs (resting eggs). Test concentrations that resulted in effects significantly different from controls were determined by Dunnetts test.

3 Results

FSH had no significant effect on asexual population growth rate (r); however, LH had strongly significant positive effect on the development of females ($p < 0.05$; MS = 0.00397, DF = 35.0) and males ($p < 0.05$; MS = 0.03041, DF = 35.0) (Figs. 1 and 2). LH and FSH enhanced the development of asexual females with parthenogenetic eggs ($p < 0.05$; Figs. 3 and 4). In addition, LH and FSH

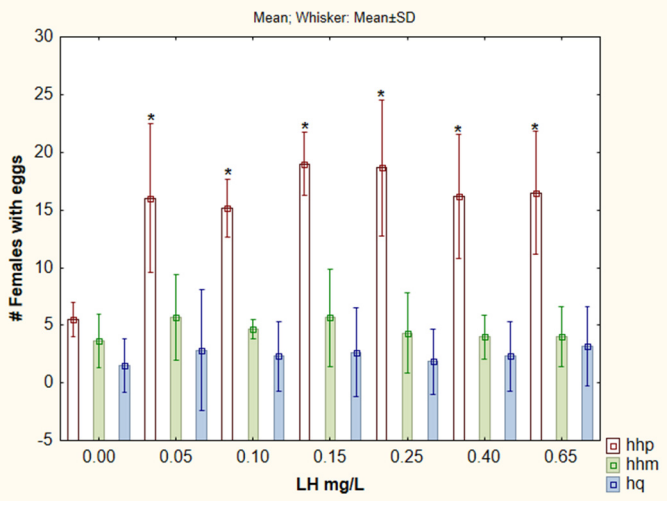

Fig. 3. Effects of LH on the production of asexual females with parthenogenetic eggs (hhp), sexual females with unfertilized sex eggs (hhm: originating males), and sexual females with fertilized sexual eggs or cysts (hq) in *Brachionus calyciflorus*. Vertical lines on columns indicate standard deviations. Asterisks (*) represent significant differences with respect to control ($p > 0.05$). $N = 6$.

resulted in a significant increase in the production of parthenogenetic eggs ($p < 0.05$ (Figs. 5 and 6). However, both hormones had no significant effect ($p > 0.05$) on the production of sexual females and their respective sexual eggs (Figs. 3–6). LH and FSH produce organisms with deformations: two abnormal females were found (Fig. 7), one at an exposure concentration of 0.0001 mg/L, and the other at an exposure concentration of 0.0003 mg/L, out of a

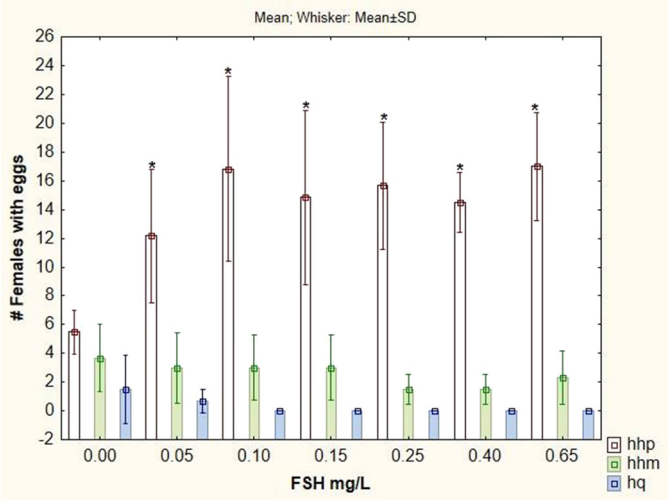

Fig. 4. Effects of FSH on the production of asexual females with parthenogenetic eggs (hhp), sexual females with unfertilized sexual eggs (hhm: originating males), and sexual females with fertilized sexual eggs or cysts (hq) in *Brachionus calyciflorus*. Vertical lines on columns indicate standard deviations. Asterisks (*) represent significant differences with respect to control ($p > 0.05$). $N = 6$.

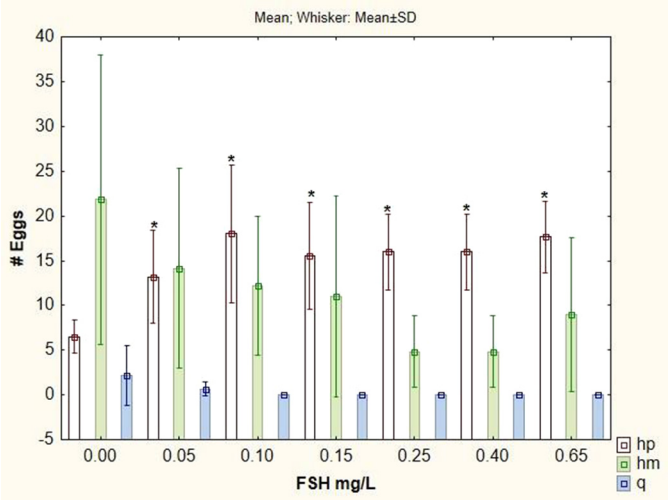

Fig. 6. Effects of FSH on the total production of asexual parthenogenetic eggs (hp), unfertilized sex eggs (hm: originating males), and fertilized sex eggs or cysts (q) in the rotifer *Brachionus calyciflorus*. Vertical lines on columns indicate standard deviations. Asterisks (*) represent significant differences with respect to control ($p > 0.05$). $N = 6$.

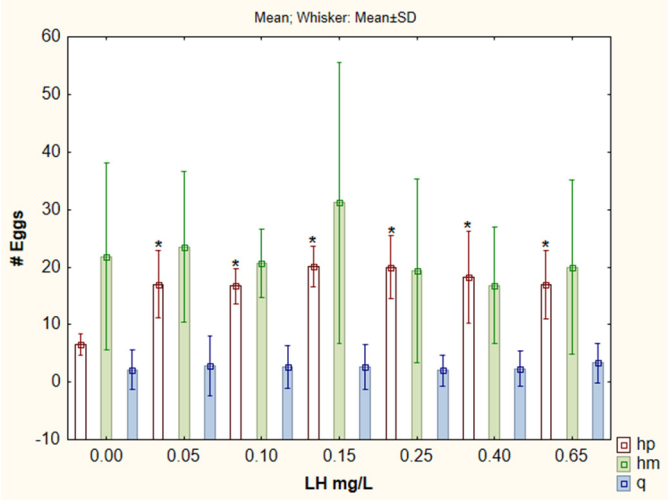

Fig. 5. Effects of LH on the production of asexual parthenogenetic eggs (hp), unfertilized sex eggs (hm: originating males), and fertilized sex eggs or cysts (q) in *Brachionus calyciflorus*. Vertical lines on columns indicate standard deviations. Asterisks (*) represent significant differences with respect to control ($p > 0.05$). $N = 6$.

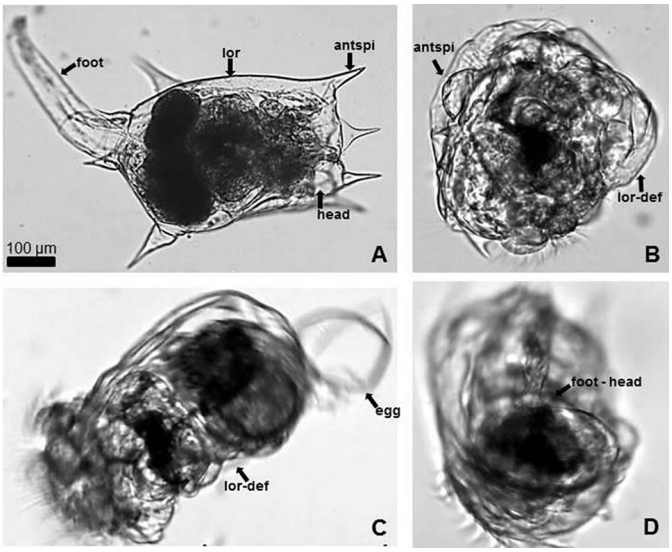

Fig. 7. Morphological alterations in females in the rotifer *Brachionus calyciflorus* upon the addition of LH and FSH to culture medium. (A) Normal female, (B) abnormal female produced by LH exposure, (C, D) abnormal female produced by FHS exposure; (C) the presence of deformed eggs and (D) the foot joined to the corona of cilia. lor = lorica, antspi = anterior spine, lor-def = lorica deformed.

total of 612 females (normal + abnormal) produced in the 4-day reproductive assay. The two abnormal females constitute 0.33% of the total population studied. At the end of the experiment (4 days), they were isolated from the pooled contents of a 24-well polystyrene plate in a total volume of 1 ml of EPA medium, and fed with the alga *N. oculata*. Both abnormal females were able to live only 48 h under bioclimatic chamber conditions at a temperature of 25 ± 2 °C, and did not reproduce.

FSH also resulted in the production of abnormal females, although these abnormal females were able to reproduce. Six abnormal females from a total of 549 females (normal + abnormal) were produced in the 4-day reproductive assay. The six abnormal females represent 1.09% of the total population studied. Abnormal females were found at the following

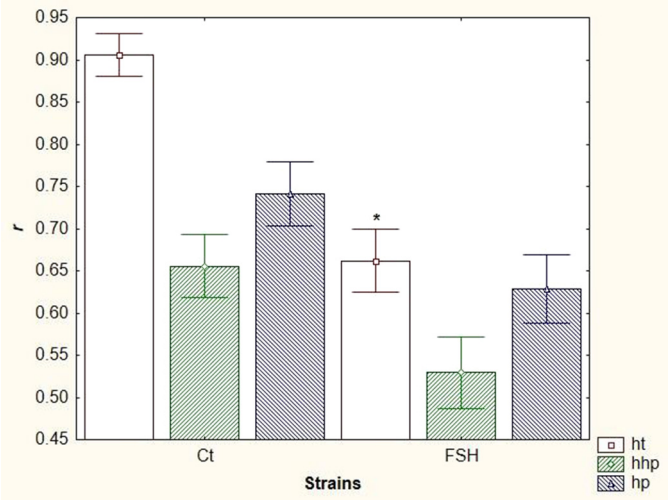

Fig. 8. Effects of FSH on the population growth rate of females (ht), females with parthenogenetic eggs (hhp), and parthenogenetic eggs (hp) in the rotifer *Brachionus calyciflorus*. The concentration of FSH used was 0.4 mg/L. Population growth rate (r) is defined as offspring per female per day. Vertical lines on columns indicate standard deviations. Asterisks (*) represent significant differences with respect to control ($p > 0.05$). $N = 6$.

concentrations: one at 0.05 mg/L, one at 0.2 mg/L, two at 0.3 mg/L, and two at 0.4 mg/L. All the abnormal females were isolated from the pooled contents of a 24-well polystyrene plate in a total volume of 1 ml of EPA medium, and fed with the alga *N. oculata*.

Five of these abnormal females were able to live only 48 h under bioclimatic chamber conditions at a temperature of 25 ± 2 °C. One of the abnormal females produced upon exposure to 0.4 mg/L FSH presented with abnormalities in the foot: it was observed to be stuck to the crown of cilia and could not be detached, which suggests that it is fused by the curvature of the body of the rotifer (observations at 20× and 40×). Only one abnormal female produced upon exposure to 0.4 mg/L FSH was able to reproduce 72 h after being isolated, and produced a parthenogenetic egg from which a perfectly normal female (parthenogenetic reproduction) hatched (Fig. 7C). This normal female was isolated in a well of a 24-well polystyrene plate in a total volume of 1 ml of EPA medium, and fed the alga *N. oculata*. From this culture, it was possible to obtain a clonal culture under bioclimatic chamber conditions. This strain was maintained for 15 days, and 105 cysts were obtained. This clonal culture was cultured for 2 months. The hatching percentage of the 105 cysts was 58.10%, while those of the control group was 60% ($n = 120$). The organisms hatched from the cysts were given a 4-day reproductive trial, without exposure to FSH, and the results were as follows: a decrease in the production of females was observed, and this decrease is significant compared to control (Fig. 8). The control corresponds to organisms hatched from cysts obtained in standard cultures under laboratory conditions. There were no significant differences in the production of females with parthenogenetic eggs (hhp), and the production of parthenogenetic eggs (hp). No production of males and cysts was observed during the 2 months.

4 Discussion

The addition of FSH and LH to reconstituted water or to the culture medium of rotifers has an effect on sexual and asexual reproduction: LH induces the production of males, whereas FSH does not; furthermore, the abnormal cohort of rotifers descended from LH does not reproduce while that of FSH does, under experimental conditions. LH does produce cysts while FSH does not, during chronic exposure for 4 days. Yang and Snell (2010) reported a decrease in cyst production when *B. calyciflorus* was exposed to progesterone and estrogens added to the medium at the same time. On the other hand, Snell and DesRosiers (2008) reported increased production of cysts, hatching of asexual and sexual eggs, and copula when progesterone was added to the culture medium of *B. calyciflorus*.

The endocrine system in rotifers remains unknown. However, great advances have been made in ecotoxicology and ecophysiology studies (Gallardo *et al.*, 1997; Gallardo *et al.*, 1999; Gallardo *et al.*, 2000; Pérez-Legaspi *et al.*, 2008; Alvarado-Flores *et al.*, 2009; Stout *et al.*, 2010; Yang and Snell, 2010). Scientific evidence suggests that GnRH stimulates the secretion of LH and FSH, because GnRH increases population growth in the rotifers *B. calyciflorus* and *B. plicatilis* (Gallardo *et al.*, 1999). Therefore, we suggest, based on our results, that FSH promotes parthenogenesis (mitosis) and in turn increases the production of estrogens that stimulate the release of large amounts of LH; subsequently, LH favors the formation of haploid eggs that develop into males, resulting in the observed high production of males in the 4-day exposure assay.

All these mechanisms of hormonal action in rotifers are regulated and coordinated by important glandular structures such as the vitellarium and cerebrum (Snell and DesRosiers, 2008; Yang and Snell, 2010). EDCs are substances that have the ability to negatively affect the function of endocrine systems; in other words, they disturb reproductive functions (Vilela *et al.*, 2018). Therefore, EDCs could cause morphological alterations from the embryonic stage and during the life cycle, according to our results and reports in literature. For example, progesterone is a steroidal hormone that plays a key role in the reproduction of many vertebrates (Graham and Clarke, 1997). Moreover, the progesterone receptor in rotifers is located in the ovaries, vellum, oviducts, parthenogenetic eggs, the seminal vesicle, in the intestine, and a large part of the spermatic duct (Stout *et al.*, 2010). LH has been detected in the corona of *B. calyciflorus* males (Alvarado-Flores *et al.*, 2009), suggesting the presence of a hormone signaling cascade in rotifers that regulates sexual reproduction (Snell and DesRosiers, 2008).

In rotifers, reproductive effects by hormone (EDCs) have been reported (Preston *et al.*, 2000; Dahms *et al.*, 2011). In general, EDCs had no effect on asexual reproduction, but reduced sexual reproduction. For example, exposure of *B. calyciflorus* to pesticides like fenitrothion, a major endocrine disruptor and an antagonist of the androgen receptor, results in the production of females that have longer reproduction periods and produce more offspring than controls, and also results in a reduced number of cysts (Lv *et al.*, 2010). Similarly, it has been observed that pentachlorophenol and chlorpyrifos reduce sexual reproduction

in the rotifer *B. calyciflorus*; in the same species, diazinon, fenitron, isoprothiolane, and methoprene increase the production of sexual eggs, and nonylphenol inhibits fertilization in females (Zou, 2003).

B. calyciflorus is very sensitive to LH and FSH exposure: morphological alterations are observed in parthenogenetic females (0.33% for LH and 1.09% for FSH), and the number of males increases. These results are relevant from the physiological and ecological point of view, due to the morphological changes and the increase in males in rotifers. Both hormones alter the reproductive, behavioral, and structural functions of rotifers. In fact, in the presence of cholesterol, LH synthesizes androstenedione, and later testosterone. In turn, estradiol and estrone are synthesized (Bachmann *et al.*, 2015) from both androstenedione and testosterone by the action of the aromatase enzyme and upon FSH stimulation. Furthermore, Yang and Snell (2010) reported that testosterone, progesterone, estrogen, or similar molecules are used by the rotifer *B. calyciflorus* to regulate sexual and asexual reproduction. Therefore, the discharge of chemical substances, with the potential for endocrine disruption in water where rotifers reside, is an environmental hazard. For example, in the hospital, synthetic hormones are frequently used, like follitropin, estrogens, and progesterone, and unfortunately, many hormones are sold by pharmacies (Thanh Thuy and Nguyen, 2013). Consequently, the intensity and frequency in use of synthetic hormones will become hazard pollutants for aquatic life.

As a result, our study of the effects of LH and FSH on *B. calyciflorus* contributes to broaden our existing knowledge on sexual reproduction, in particular the inhibition of sexual reproduction in males. However, it mainly serves to evaluate the potential adverse effects of EDCs in rotifers. In addition, this study provides quantitative information on the induction of deformations (teratology in rotifers) and infertility due to exposure to hormones added to the culture medium. In conclusion, rotifers are particularly sensitive to LH and FSH, and their response at histological and physiological levels offers an effective biological indicator to understand the hazards of EDCs (Preston *et al.*, 2000).

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