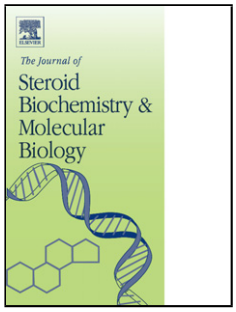

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The key action of estradiol and progesterone enables GnRH delivery during gestation in the South American plains vizcacha, *Lagostomus maximus*.

Running head: GnRH modulation by estrogen and progesterone in the vizcacha.

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Highlights

- Pharmacological doses of progesterone and estradiol results in an inhibition of hypothalamic GnRH expression.
- Physiological doses of progesterone and estradiol showed a differential effect over GnRH pulsatile delivery frequency or genomic GnRH expression.
- The modulation of GnRH delivery and expression would be subjected to different levels of action of steroid hormones.

- A short-term effect of E2 would modulate the frequency of GnRH delivery pattern whereas a long-term effect of E2 would modify the GnRH mRNA expression
- The fine action of E2 and P4 constitute the key factor to enable the hypothalamic activity during the pregnancy of this mammal.

Abstract

The South American plains vizcacha, *Lagostomus maximus*, is the only mammal described so far that shows expression of estrogen receptors (ERs) and progesterone receptors (PRs) in gonadotropin-releasing hormone (GnRH) neurons. This animal therefore constitutes an exceptional model for the study of the effect of steroid hormones on the modulation of the hypothalamic-pituitary-ovarian (HPO) axis. By using both *in vivo* and *ex vivo* approaches, we have found that pharmacological doses of progesterone (P4) and estradiol (E2) produced an inhibition in the expression of hypothalamic GnRH, while physiological doses produced a differential effect on the pulsatile release frequency or genomic expression of GnRH. Our *ex vivo* experiment indicates that a short-term effect of E2 modulates the frequency of GnRH release pattern that would be associated with membrane ERs. On the other hand, our *in vivo* approach suggests that a long-term effect of E2, acting through the classical nuclear ERs-PRs pathway, would produce the modification of GnRH mRNA expression during the GnRH pre-ovulatory surge. Particularly, P4 induced a rise in GnRH mRNA expression and protein release with a decrease in its release frequency. These results suggest different levels of action of steroid hormones on GnRH modulation. We conclude that the fine action of E2 and P4 constitute the key factor to enable the hypothalamic activity during the pregnancy of this mammal.

Abbreviations

ANOVA	one-way analysis of variance
ARC	arcuate nucleus
BSA	bovine serum albumin
E2	estradiol
ECAS	Estación de Cría de Animales Silvestres
ERs	estrogen receptors

ERα	estrogen receptor alpha
FSH	follicle-stimulating hormone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GnRH	gonadotropin-releasing hormone
hCG	human chorionic gonadotropin
HP	hypothalamic-pituitary
HPO	hypothalamic-pituitary-ovarian
KRB	Krebs Ringer buffer
LH	luteinizing hormone
NP	non-pregnant
OVX	ovariectomized
P4	progesterone
PFA	neutral-buffered paraformaldehyde
PMSG	pregnant mare's serum gonadotropin
POA	preoptic area
PRs	progesterone receptors
RIA	radioimmunoassay
SD	standard deviation
SHAM	surgery simulated non-pregnant
SNP	stimulated non-pregnant
SON	supraoptic nucleus
VMN	ventromedial nucleus

Keywords: GnRH, estradiol, progesterone, pregnancy, ovariectomy, vizcacha.

1. Introduction

Pubertal development and adult reproductive function depend on the activation of the hypothalamic-pituitary-ovarian (HPO) axis. In most species, gonadotropin-releasing hormone (GnRH), a decapeptide involved in the modulation of the HPO axis, is synthesized in the hypothalamus by a discrete specialized group of neurons scattered throughout the preoptic area (POA), the ventromedial nucleus (VMN) and the arcuate nucleus (ARC) (Urbanski *et al.* 1991, Urbanski *et al.*1992, Silverman & Witkin 1994). The majority of GnRH neurons project their processes towards the median eminence (ME), releasing GnRH into the hypothalamic-pituitary portal circulation, that transports the hormone to the anterior pituitary gland where it binds to its specific receptor and modulates gonadotropin synthesis and delivery (Krey & Silverman 1978, Silverman *et al.* 1987, Silverman & Witkin 1994, Witkin *et al.* 1995, Yin *et al.* 2009a, Yin *et al.* 2009b). As the central regulator of fertility in mammals, GnRH is released in discrete pulses separated by periods of little to no secretion, from puberty up to menopause, except during pregnancy (Belchetz *et al.* 1978). This mode of secretion sensitizes the pituitary gonadotrophs to GnRH stimulation and regulates gonadotropin gene expression (Wetsel *et al.* 1992).Variations in the pulsatile pattern of GnRH release differentially modulates the synthesis and secretion of the two pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), that influence gonadal gametogenesis, folliculogenesis and steroidogenesis (Wildt *et al.* 1981, Marshall & Griffin 1993). Low GnRH pulse frequency favors FSH release whereas high pulse frequency stimulates the release of LH (Wildt *et al.* 1981, Gharib *et al.* 1990, Burger *et al.* 2008, Ciccone *et al.* 2010). Although the pulsatile secretion of GnRH is an intrinsic property of hypothalamic GnRH neurons, attributed to specific mechanisms of spontaneous electrical activity, its pulsatile delivery frequency and amplitude is under modulation of a complex network of molecules (Krsmanovic *et al.* 2009). One of the classical pathways of GnRH modulation includes the feedback produced by the gonadal steroid hormones progesterone (P4) and estradiol (E2) (Yen *et al.* 1975, Goodman & Karsch 1980, Knobil 1980, White *et al.* 2007, Thackray *et al.* 2009, Yin *et al.* 2009a, Yin *et al.* 2009b).

Although most mammals show inhibition of the HPO axis during gestation, we have recently described that the South American plains vizcacha (*Lagostomus maximus*), a hystricognathe caviomorph rodent inhabiting the southern area of the Neotropical region, especially the Pampean region of Argentina (Jackson *et al.* 1996), displays reactivation of the reproductive

axis at mid-gestation (Dorfman *et al.* 2013, Fraunhoffer *et al.* 2017, Inserra *et al.* 2017) among other exceptional reproductive traits such as the highest ovulation rate, so far recorded for a mammal, up to 800 oocytes per estrous cycle (Weir 1971a, Weir 1971b), natural selective and sequential resorption of the anteriorly implanted fetuses (Weir 1971a), and suppression of apoptosis-dependent follicular atresia driven through an over-expression of the anti-apoptotic *BCL2* gene and a basal or absent expression of pro-apoptotic *BAX* gene, both in the developing and adult ovary (Jensen *et al.* 2006, Leopardo *et al.* 2011, Inserra *et al.* 2014). We hypothesized that the reactivation of the HPO axis during gestation is enabled by a fine equilibrium in the neuroendocrine environment of the pregnant vizcacha that makes possible follicular maturation and development of a new set of secondary corpora lutea that provides the hormonal boost necessary to get pregnancy to term. This event correlates with an increased expression of hypothalamic GnRH, estrogen receptor alpha (ERα) and progesterone receptors (PRs), despite increased and sustained levels of serum P4, E2 and LH (Dorfman *et al.* 2013, Fraunhoffer *et al.* 2017, Inserra *et al.* 2017, Proietto *et al.* 2019). Finally, we have also shown that GnRH neurons of POA and supraoptic nucleus (SON) express ERα and PRs, suggesting a direct action of E2 and P4 to assure GnRH synthesis and delivery during pregnancy (Dorfman *et al.* 2013, Inserra *et al.* 2017).

The aim of this study was to evaluate the involvement of E2 and P4 in the modulation of hypothalamic GnRH synthesis and release in this species with this particular reproductive strategy. In order to elucidate this matter, we employed both *in vivo* and *ex vivo* approaches, exposing the hypothalamus to physiological and pharmacological doses of E2 and P4, and agonists and antagonists of their specific receptors.

2. Materials and methods

2.1 Ethics

All experimental protocols concerning animal handling were conducted in accordance with the guidelines published in the National Institutes of Health (NIH) guide for the care and use of laboratory animals (National Research Council 2011), and were reviewed and approved by the Institutional Committee on Use and Care of Experimental Animals (CICUAE) from Universidad Maimónides, Argentina (Resolution Nº 16/14).

2.2 Animals

Adult non-pregnant (NP) female plains vizcachas (n=80) were captured from a resident natural population at the *Estación de Cría de Animales Silvestres* (ECAS), Villa Elisa, Buenos Aires, Argentina, using live-traps located at the entrance of burrows. Appropriate procedures were performed to minimize the number of animals used. Captures were planned according to the natural reproductive cycle, as described by Llanos & Crespo (1952), and our own expertise in the field (Jensen *et al.* 2006, Jensen *et al.* 2008, Dorfman *et al.* 2011, Espinosa *et al.* 2011, Leopardo *et al.* 2011, Dorfman *et al.* 2013, Halperin *et al.* 2013, Inserra *et al.* 2014, Charif *et al.* 2016, Dorfman *et al.* 2016, Charif *et al.* 2017, Fraunhoffer *et al.* 2017, Inserra *et al.* 2017, Leopardo & Vitullo 2017, Giacchino *et al.* 2018, Leopardo *et al.* 2018, Proietto *et al.* 2018, Gariboldi *et al.* 2019, Proietto *et al.* 2019, Schmidt *et al.* 2019). All animals ranged from 2.5 to 3.5 years old as determined by the dry lens weight, according to Jackson (1986). Animals were housed under a 12:12 hour low-light cycle to simulate their natural light exposure (low light of 12W followed by moon light) at 22 ± 2°C constant room temperature, with food and tap water *ad libitum*.

2.3 Experiment 1: GnRH expression during the activation of hypothalamic-pituitary axis

In order to obtain animals with active hypothalamic-pituitary (HP) axis, 15 NP females were anaesthetized by intramuscular injection of 6.66mg/kg body weight ketamine chlorhydrate (Holliday Scott S.A., Buenos Aires, Argentina) and 0.3mg/kg body weight xylazine chlorhydrate (Richmond Laboratories, Veterinary Division, Buenos Aires, Argentina), and bilaterally ovariectomized (OVX) through a single dorsal incision. Tramadol (1mg/kg, Algen 20, Laboratorios Richmond S.A., Argentina) was administered for pain management and penicillin G (10,000IU/kg procaine benzyl penicillin + 10,000IU/kg benzathine benzyl penicillin + 16,000IU/kg dihydro-streptomycin sulfate, Fort-E-Pen, Laboratorios Brouwer S.A, Argentina) to prevent infections.

OVX females were randomly divided into three groups. The fifth day post-surgery, females were treated with intramuscular pharmacological doses of E2 (OVX+E2; n=5, 1mg/kg/day, Laboratorios Burnet S.A., Argentina), P4 (OVX+P4; n=5, 5mg/kg/day, Laboratorios Burnet S.A., Argentina) or vehicle (OVX; n=5, 1ml/kg/day) during five consecutive days. Five SHAM surgery animals were used as control group. Females were sacrificed the day followed to the last injection (Figure 1A). The pharmacological doses of E2 and P4 employed were calculated in order to produce a serum concentration of at least ten times higher than normal serum concentration of non-pregnant vizcachas, as previously described (Dorfman *et al.* 2013, Fraunhoffer *et al.* 2017).

2.4 Experiment 2: GnRH expression during induced luteal phase

In order to study hypothalamic activity during the luteal phase, 15NP females were synchronized (SNP) by inducing ovulation, as previously described (Charif *et al.* 2016, Proietto *et al.* 2019). Briefly, females were injected intramuscularly with pregnant mare's serum gonadotropin (PMSG) (250IU/day, Novormon 5000, Syntex, Argentina) during three consecutive days, followed by an intramuscular administration of human chorionic gonadotropin (hCG) (1000IU, Ovusyn 5000, Syntex, Argentina) at the fourth day. Five additional NP females were injected with vehicle and used as control group.

SNP females were randomly divided into three groups and treated with pharmacological doses of E2 (SNP+E2; n=5; 1mg/kg/day, Laboratorios Burnet S.A., Argentina), P4 (SNP+P4; n=5; 5mg/kg/day, Laboratorios Burnet S.A., Argentina) or vehicle (SNP; n=5) during five consecutive days. Females were sacrificed 14 days after the first injection of PMSG (Figure 1B). The presence of ovulatory stigmata at sacrifice was considered as inclusion criteria. Ovaries of all females were removed and fixed in cold 4% neutral-buffered paraformaldehyde (PFA) (Sigma Aldrich Inc., St. Louis, Missouri, USA) for histological inspection of the ovulatory status (corpora lutea and follicle development) by hematoxylin-eosin staining (Figure 2).

2.5 Experiment 3: GnRH expression and pulsatile delivery

In order to analyze the involvement of E2 and P4 in GnRH pulsatility, 40 NP females were synchronized by PMSG and hCG treatment, as described above. Animals were sacrificed 14 days after the first PMSG injection (Figure 1C). Hypothalamic explants were incubated during 6 hours with different combinations of E2, P4, and ER and PR agonists and antagonists (Table 1), at concentrations selected from previous reports in rats, mice, ewes and cancer cell lines (Kraichely *et al.* 2000, Mattheus *et al.* 2006, Arreguin-Arevalo *et al.* 2007, Hu *et al.* 2008, Ng *et al.* 2009, Serova *et al.* 2010, Clipperton-Allen *et al.* 2011, Kuo *et al.*2011, Lattrich *et al.* 2014). GnRH released to the incubation medium was measured by radioimmuneassay (RIA).

2.6 Tissue collection

Animals were anaesthetized by the intramuscular injection of 13.5mg/kg body weight ketamine chlorhydrate (Holliday Scott S.A., Buenos Aires, Argentina) and 0.6mg/kg body weight xylazine chlorhydrate (Richmond Laboratories, Veterinary Division, Buenos Aires, Argentina). Blood samples were taken by puncture in the inferior vena cava. After bleeding, animals were sacrificed by an intracardiac injection of 0.5ml/kg body weight of Euthanyl^{TM} (Sodic Pentobarbital, Sodic Diphenilhidanthoine, Brouwer S.A., Buenos Aires, Argentina). Brains were rapidly removed and the whole hypothalamus was dissected out following the anterior and lateral borders of the optic chiasm, the anterior border of the mammillary bodies and approximately 4mm depth, as previously described (Dorfman *et al.* 2103, Charif *et al.* 2016, Charif *et al.* 2017, Inserra *et al.* 2017). The right halves of the hypothalami were used to evaluate specific mRNA content whereas the left halves were used to evaluate protein GnRH content. Both left and right halves were immediately frozen in dry ice and stored at -80°C for RNA and protein analysis. In order to analyze GnRH pulsatile release, whole hypothalami were placed in gelatin pre-coated tubes with 500µl of Krebs-Ringer buffer (KRB) (115mM NaCl, 4.7mM KCl, 1.2mMKH_2PO4, 1.2mM $MgSO_4$, 2.56mM $CaCl_2$ and 20mM $NaHCO_3$; pH 7.4) supplemented with 0.1% bovine serum albumin (BSA), 25mM glucose and 16mM HEPES, as previously described (Charif *et al.* 2016). Surgeries were developed in coordination with others members of our group who use other organs of reproductive relevance such as mammary glands, pituitary glands, adrenal glands, pineal glands, as well as muscle, liver and kidney.

2.7 Serum progesterone and estradiol determination

Serum E2 and P4 content was determined by ELISA, as previously described (Dorfman *et al.* 2013, Charif *et al.* 2017, Inserra *et al.* 2017). Briefly, blood samples were centrifuged for 15 minutes at 3,000rpm; serum fractions were aliquoted and stored at -80ºC. Estradiol ELISA Kit (EIA-2693, DRG Int., Germany) or the Progesterone ELISA Kit (EIA-1561, DRG Int., Germany) were used to determine E2 and P4 serum levels, respectively, according to the manufacturer's instructions. Direct solid phase enzyme immunoassays that detect a range of 16-2000pg/ml of E2 or 0.18-40ng/ml of P4 were developed. Intra- and inter-assays coefficients of variation were 6.8% and 10.3%, respectively for E2 and 7.1% and 10.7% for P4. The absorbance of the solutions, measured at 450nm (µQuant Microplate Spectophotometer, Bio-tek Instruments Inc., Winooski, Vermont, USA), was inversely related to the concentration of E2 or P4 in the sample. E2 and P4 content was referred to the respective calibration curves.

2.8 RIA for serum LH detection

Serum LH content was determined by RIA with kits from the National Hormone and Pituitary Program, National Institute of Diabetes, Digestive and Kidney Diseases, USA, as previously described (Dorfman *et al.* 2013, Inserra *et al.* 2017, Proietto *et al.* 2019). Results were expressed in relation to rat LH standards using: r-LH-I10, reference preparation rat LH-RP-3 (AFP7187B) and anti-rat LH-S11 (AFPC697071P) (Catalano *et al.* 2010). Assay sensitivity was 0.31ng/ml. Intra- and inter-assay coefficients of variation were 7.0% and 11.2%, respectively. A pool of pituitaries of high LH content was serially diluted to prepare the vizcacha curve. Its parallelism with the rat standard curve was confirmed.

2.9 RNA isolation and quantitative polymerase chain reaction (qPCR)

In order to extract total hypothalamic RNA, tissues were homogenized with TRIzol (Invitrogen, California, USA), according to the manufacturer's instructions, as previously described (Charif *et al.* 2017, Inserra *et al.* 2017). Its concentration was quantified by absorption at 260nm (Genequant, Amersham Biosciences, England) and its integrity confirmed in a 1% agarose (Genbiotech, Argentina) in Tris (0.09M), boric acid (0.045M), EDTA (0.05M) (TBE) buffer gel (pH 8.3) when the presence of S28 and S18 rRNA subunits were observed. Three µg of total RNA was treated with 1µl DNaseI (Invitrogen, California, USA) in 1µl 10X DNase Reaction Buffer (Invitrogen, California, USA) for 30 minutes at 37°C, and the reaction was stopped with 1µl EDTA 50mM (Invitrogen, California, USA) for 10 minutes at 65°C. The RNA was reverse-transcribed into first-strand cDNA using 1.5µl random hexamer primers 50µM (Applied Biosystems, California, USA), 200U reverse transcriptase (RevertAid™ M-MuLV, Fermentas, Massachusetts, USA), 4µl First Strand Buffer 5x (Fermentas, Massachusetts, USA), 2µl dNTP mixture 10mM (Invitrogen, California, USA) and 0.5µl RNase inhibitor (Ribolock™, Fermentas, Massachusetts, USA), at a 20µl final volume reaction. The reverse transcriptase was omitted in control reactions where the absence of PCR-amplified cDNA indicated the isolation of RNA free of genomic DNA. Reverse transcription reaction was carried out at 72ºC for 10 minutes followed by 42°C for 60 minutes and stopped by heating at 70°C for 10 minutes. cDNA was stored at -20ºC until use. Three micrograms of cDNA was mixed with 6µl SYBR Green PCR Master Mix (Applied Biosystems, United Kingdom) for qPCR using 0.3µM forward and reverse oligonucleotide primers. Primer sequences and cycling parameters for each product are shown in Table 2. These primers were previously employed in vizcacha (Gonzalez *et al.* 2012, Dorfman *et al.* 2013, Charif *et al.* 2016, Charif *et al.* 2017, Fraunhoffer *et al.* 2017, Inserra *et al.* 2017). Quantitative measures were performed using a Stratagene MPX500 cycler (Stratagene, California, USA). Data were collected from the threshold value, taken at the 72ºC extension phase, continuously stored during reaction and analyzed by the complementary computer

software (MxPro3005P v4.10 Build 389, Schema 85, Stratagene, California, USA). To confirm the specificity of the signal, the results were validated based on the quality of dissociation curves generated at the end of the qPCR runs. For each target gene, the relative quantitation of gene expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene. For the assessment of quantitative differences in the cDNA target between samples, the mathematical model of Pfaffl (2001) was applied. An expression ratio was determined for each sample by calculating $(E_{target})^{\Delta Cq(target)}/(E_{GAPDH})^{\Delta Cq(GAPDH)}$, where E is the efficiency of the primer set and ΔCq (quantification cycle) is the difference in the threshold cycle with $\Delta Cq = Cq_{(normalization\ cDNA)} - Cq_{(experimental\ cDNA)}$. The amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log (ng cDNA) per reaction vs. Cq value ($E = 10^{-(1/slope)}$). Efficiencies of 2.0 ± 0.1 were considered optimal. Each sample was analyzed in triplicate along with non-template controls to monitor contaminating DNA. Purity of the amplified products was confirmed by 2% agarose gel electrophoresis (Biodynamics, Buenos Aires, Argentina). The presence of the amplified sequence was detected with an UV trans-illuminator (Labnet DyNA Light TM-26, USA). Corresponding gel bands were excised and purified with the Min Elute Gel Extraction kit (Qiagen, Hilden, Germany). To confirm GnRH, ERα, PR and GAPDH identities purified products were sequenced with a 3130xl Genetic Analyzer (Applied Biosystems, California, USA) by the Genomic Unit of the Biotechnology Institute, *Instituto Nacional de Tecnología Agropecuaria* (INTA), Buenos Aires, Argentina. Using the Bioedit software (Ibis Biosciences, California, USA) the obtained sequences of ERα, PR and GnRH were aligned together with the corresponding sequences published for other species and the percentage of homology determined with the DNA Single Polymorphism software (DNAsp version 5.0) (Rozas 2009); see Table 2.

2.10 RIA for GnRH detection

Hypothalamic GnRH content or GnRH delivered by hypothalamic explants was measured by RIAs previously described in mouse (Di Giorgio *et al.* 2013) and vizcacha (Dorfman *et al.* 2013, Charif *et al.* 2016, Charif *et al.* 2017, Inserra *et al.* 2017). For hypothalamic detection of GnRH, tissues were homogenized in 100µl of HCl 0.1N, centrifuged for 30 minutes at 13,000g and supernatants recovered. All procedures were carried out at 4ºC. GnRH concentration was analyzed in duplicate using anti-GnRH antiserum (rabbit polyclonal HU-60 that recognizes GnRH1 with higher affinity than GnRH2, final dilution 1:50,000) (Mongiat *et al.* 2006) kindly provided by Dr. Urbanski (Division of Neuroscience, Oregon National Primate Research Center). GnRH was iodinated with ^{125}I (NEZ 033H Iodine125, Perkin Elmer, Life and Analytical Science, Waltham, Massachusetts, USA) by the chloramine-T method (Greenwood *et*

al. 1963). Intra- and inter-assay coefficient of variation was 6.8% and 10.9%, respectively. Assay sensitivity was 1.5pg. The total protein content of each sample was determined by Bradford assay (Bradford 1976). Hypothalamic GnRH content was expressed as the ratio between the value obtained by RIA and the total protein content, while GnRH released to the medium in the *ex vivo* experiments was expressed as the ratio between the value obtained by RIA and the weight of each hypothalamic explant.

2.11 Hypothalamic GnRH release

GnRH pulsatility was measured *ex vivo* as previously described in mice (Catalano *et al.* 2010) and vizcacha (Charif *et al.* 2016). Briefly, hypothalami explants were pre-incubated for 30 minutes at 37°C in 500µl of fresh KRB (control) or KRB supplemented with steroid hormones with or without the appropriate receptor agonist and antagonist, as described in Table 1. After pre-incubation, hypothalami were further incubated for 6 hours at 37°C in fresh or supplemented KRB (Table 1). During incubation, medium was collected at 7.5-minutes intervals, stored at -20°C, and replaced with fresh or supplemented KRB. In order to test tissue viability, a depolarizing concentration of potassium chloride (100mM) was added to the last tube for 30 minutes. A marked peak of GnRH release was identified. GnRH concentration was determined by RIA as described above. GnRH pulsatile parameters were determined using the computer algorithm Cluster8 developed by Veldhuis & Johnson (1986) (Pulse_XP software, http://mljohnson.pharm.virginia.edu/home.html). A 2x2 cluster configuration and a t-statistic of 2 for the up stroke and down stroke, to maintain false-positive and false-negative error rates <10%, were used as suggested by Martinez de la Escalera *et al.* (1992). GnRH pulsatile frequency and GnRH total mass delivered were informed.

2.12 Statistical analysis

Values were expressed as mean ± standard deviation (SD). All the experiments were performed by duplicate. Results were evaluated using one-way analysis of variance (ANOVA). Comparisons among groups were made by Bonferroni post-test. Statistical analysis was performed using Prism 4.0 (GraphPad Software Inc., San Diego, California, USA). Differences were considered significant when $p<0.05$.

3. Results

3.1 Experiment 1: GnRH expression during activated HP axis

Ovariectomy significantly modified serum LH, P4 and E2 levels, confirming the activation of the HP axis (Figure 3A-C). OVX treatment significantly increased LH serum levels whereas OVX+P4 and OVX+E2 treatment reverted LH to control SHAM values (Figure 3A). In addition, OVX females showed a significant decrease of P4 and E2 serum levels (Figure 3B-C). The efficiency of the pharmacological treatment with P4 and E2 was confirmed by the detection of significant increased values of serum P4 and E2 in OVX+P4 and OVX+E2 animals, respectively (Figure 3B-C). Hypothalamic GnRH mRNA and protein content showed a pattern similar to that of LH, with a significant increase in OVX animals that was reverted with the pharmacological treatment with both P4 or E2 (Figure 3D-E).

3.2 Experiment 2: GnRH expression during induced luteal phase

PMSG and hCG treatment induced significant changes in serum LH, P4 and E2 levels (Figure 4A-C). Accordingly, ovulatory stigmata and abundance of corpora lutea formation were observed in ovaries of all SNP animals, confirming the luteal phase (Figure 2). SNP females showed significantly increased levels of serum LH, whereas SNP animals treated with P4 or E2 showed significantly lower LH levels, with values slightly over those of NP animals (Figure 4A). In addition, the high levels of serum P4 and E2 detected in pharmacologically treated SNP animals confirmed the efficiency of the treatment (Figure 4B-C). Hypothalamic mRNA and protein content of GnRH decreased significantly when PMSG+hCG treatment was applied, regardless of P4 or E2 administration (Figure 4D-E).

3.3 Experiment 3: ex vivo GnRH pulsatile release and mRNA expression

In order to evaluate P4 and E2 involvement in GnRH release, hypothalamic explants of NP females, whose estrus cycles were synchronized by PMSG+hCG treatment, were incubated

with: a) P4 and/or the PR antagonist RU486, or b) E2 or combinations of ERα and ERβ agonists and antagonists, as shown in Table 1. Experiment 3a showed that P4 significantly decreased GnRH pulsatile release frequency from 5 to 3 pulses (Figure 5A) and flattened the pulsatile pattern related to the other evaluated groups (Figure 5C-F). On the contrary, both P4 and RU486 treatment significantly increased the total GnRH mass released compared to control hypothalamic explants (Figure 5B). On the other hand, experiment 3b depicted a significant induction of GnRH pulsatile frequency by E2 (Figure 6A), with no alteration of the total GnRH mass released (Figure 6B). However, the total GnRH mass released significantly increased when ERα or ERβ were singly induced (Figure 6B) whilst, in both cases, the pulsatile patterns were flatter than those obtained by E2 supplement or even without any supplementation (Figure 6C-F). In all the cases, the supplementation of the incubation media with KCl during the final 30 minutes of the experiment showed a pronounced GnRH delivery indicating the viability of all the analyzed tissues throughout the experiment (Figures 5C-F and 6C-F).

Finally, ERα, PR and GnRH mRNA content in the hypothalamic explants was analyzed at the end of both experiments (Figure 7). ERα mRNA content was significantly increased in relation to control in both E2 treatment and specific ERα and ERβ agonist treatment (Figure 7A). However, neither PR nor GnRH mRNA content was altered in these groups (Figure 7B-C). ERα mRNA content did not change when hypothalamic explants were supplemented with P4 or RU486 (Figure 7D). Moreover, PR mRNA levels were significantly increased by P4 treatment in relation to control and RU486 treatment, confirming the positive feedback exerted by P4 over its own receptor (Figure 7E). Finally, GnRH mRNA content significantly increased only in the hypothalamic explants when the PR was blocked with its antagonist RU486 (Figure 7F).

4. Discussion

The present work shows that both E2 and P4 have a key role in modulating the synthesis and delivery of pituitary LH and hypothalamic GnRH in the vizcacha, a species with a peculiar reproductive strategy. Since previous descriptions of the reproductive anatomy, histology and

physiology of the vizcacha showed species-specific features that differ markedly from most mammalian reproductive traits established by observations of murines and a few other species, this work focused on evaluating pituitary and hypothalamic activity of vizcacha subjected to different hormonal environments.

Our first approach consisted of studying LH and GnRH variations in a bilateral ovariectomy condition, with no endogenous ovarian steroid hormones. As expected, the absence of steroid hormones induced physiological conditions concordant with an active hypothalamus and pituitary gland, probably generated by the absence of the steroid negative feedback, and reflected in the significant rise of GnRH and LH. Similar observations were previously reported in other species, such as ovariectomized rats and ovariectomized or post-menopausal women (Bohm-Levine *et al.* 2019, Hussien *et al.* 2019). In OVX vizcachas treated during 5 consecutive days with E2 or P4 the overexpression of both LH and GnRH was reversed, showing lower levels of LH and GnRH than those of SHAM animals, confirming the strong inhibitory effect that these hormones exert at pituitary and hypothalamic level, as was also reported in other species, such as rats, mice and ewes (Sarkar & Fink 1980, Zoeller *et al.* 1988, Caraty *et al.* 1989, Chongthammakun & Terasawa 1993, Petersen *et al.* 1995, Spratt & Herbison 1997, Radovick *et al.* 2012).

The second approach consisted of the induction of the luteal phase by the synchronization of the HPO axis of NP females by administration of PMSG and hCG. This treatment produced high levels of serum E2 and P4 together with high levels of serum LH and low levels of GnRH. This suggests that E2 and P4 may exert a different effect at pituitary and hypothalamic levels, indicating that these organs present a different sensitivity and response to steroids. The negative feedback of E2 and P4 at hypothalamic level has been widely reported for other species, such as mice, rats, ewes and rhesus monkeys (Sarkar & Fink 1980, Zoeller *et al.* 1988, Petersen *et al.* 1995, Spratt & Herbison 1997, Caraty *et al.* 1989, Chongthammakun & Terasawa 1993). In addition, high levels of E2, P4 and LH, together with low levels of GnRH, have been previously reported for the luteal phase of the vizcacha (Dorfman *et al.* 2103, Inserra *et al.* 2107). Moreover, similarly to the results we obtained in OVX animals, the pharmacological treatment of non-pregnant vizcachas in early luteal phase with E2 or P4 produced a dramatic decrease in GnRH levels, showing the expected inhibitory effect on the hypothalamus that these steroid hormones exert.

As mentioned before, female vizcachas display some unique reproductive traits that differentiate them from other mammalian species. Among those noteworthy reproductive features, the continuous formation of pre-ovulatory follicles during the 155-day lasting pregnancy, the reactivation of the HPO axis and the formation of numerous secondary corpora lutea with oocyte retention that provides a hormonal boost at mid-gestation are highlighted (Jensen *et al.* 2008, Dorfman *et al.* 2016, Inserra *et al.* 2017). At the time of the HPO axis reactivation, GnRH

and LH expression are significantly increased, together with relatively high levels of P4 and E2 (Dorman *et al.* 2013, Inserra *et al.* 2017, Proietto *et al.* 2019). Moreover, hypothalamic GnRH neurons co-express PRs and ERs, converting the vizcacha in a valuable model to study the direct regulation of E2 and P4 over GnRH expression (Dorfman *et al.* 2013, Inserra *et al.* 2017). This contrast with the classical model of GnRH indirect regulation by ovarian hormones. In order to elucidate the direct effect that steroid hormones would exert over GnRH neurons, we employed animals with induced luteal phase and developed an *ex vivo* model of hypothalamic explants that were treated with steroid hormones or with combinations of agonists and antagonists of their specific receptors. Our results showed a rise in GnRH pulsatile frequency induced by treatment with E2 that was counterbalanced by a decrease in the total GnRH mass delivered. There is wide *in vitro* evidence of the negative estrogen regulation of GnRH synthesis, both at protein and mRNA levels, employing immortalized GnRH-producing GT1-7 hypothalamic neuron cells which express ERα and ERβ (Radovick *et al.* 2012, Kepa *et al.* 1992, Wierman *et al.* 1992, Roy *et al.* 1999, Otani *et al.* 2009). However, in our *ex vivo* model, no differences of GnRH mRNA levels were found with E2 treatment suggesting that a 6-hour treatment with E2 is enough to induce an increase in the release of the stocked GnRH protein but insufficient to produce transcriptional changes. In addition, the frequency of GnRH release induced by E2, but not when ERα or ERβ were separately activated, suggests the possible involvement of another class of ERs in this mechanism. In this way, the activation of membrane ERs, which are usually responsible for rapid and short term non-genomic modulation, such as GPR30 or nuclear ERα that translocates to the cell membrane after cleavage and associates with metabotropic glutamate receptors (mGluRs), should be considered in future experiments in order to fully understand the mechanisms of GnRH delivery induction (Takeo & Sakuma 1995, Kim *et al.* 2011a, Kim *et al.* 2011b, Levin 2011, Prossnitz & Barton 2011, Zárate *et al.* 2012, Wong *et al.* 2019).

Surprisingly, both *ex vivo* treatments used to evaluate ERα or ERβ involvement in GnRH release (the treatment combining ERα agonist with ERβ antagonist vs. the treatment combining ERα antagonist with ERβ agonist) showed a similar behavior. Previous reports suggested that ERα is crucial for the differential modulation of the positive and negative feedback loop exerted by E2, with no involvement of ERβ. Employing knockout mice for ERα and ERβ, it was demonstrated that ERα-knockout mice presented high LH levels and no ability to generate the pre-ovulatory LH surge, necessary to induce ovulation (Herbison 1998, Couse & Korach 1999, Wintermantel *et al.* 2006). Concordantly, in a previous work we showed a positive correlation between ERα protein and mRNA levels and E2 and LH serum levels throughout gestation in the vizcacha, while ERβ protein and mRNA levels remained constant (Inserra *et al.* 2017).

However, the present results show that both ERα and ERβ variants may participate in the modulation of GnRH delivery.

On the other hand, P4 produced a decrease in GnRH pulsatile frequency and this effect was reverted when PR was blocked by its specific receptor antagonist RU486. However, a rise in the total GnRH mass delivered was registered by P4 treatment. In a previous report we described a correlation between GnRH, P4 and PR levels and the presence of accessory corpora lutea at mid-gestation suggesting a possible role of PR in the reactivation of the HPO axis (Dorfman *et al.* 2013). Despite that P4 treatment was able to induce PR mRNA expression, it failed to induce GnRH mRNA expression, probably because a 6-hour treatment was not enough to produce genomic effects. The induction of PRs is necessary for the successful release of GnRH. This was proved in PR gene knock-out (PRKO) mice treated with E2, where GnRH and LH surges were absent (Chappell *et al.* 1997, Chappell *et al.* 1999), and in rats treated with PR antagonist or with intracerebroventricular injection of PR antisense oligonucleotides (Chappell & Levine 2000). All these data suggest a genomic key role for PR in the preovulatory GnRH surge. Considering that E2 induces expression of PR, and that this event is obligatory for GnRH self-priming (Chappell & Levine 2000), the unchanged levels of PR mRNA in E2-induced hypothalamic explants clearly explain that 6 hours of estrogens were not enough to induce PR genomic changes and consequently GnRH mRNA variations.

5. Conclusions

The combined results of the three experiments performed in this study suggest different levels in the modulation of steroid hormones over GnRH delivery. Based on our results, we propose a short-term effect of E2 that modulates the frequency of GnRH release pattern, as shown in the 6-hour *ex vivo* experiments, probably associated with membrane ERs; and a long-term effect of E2 acting through the classical nuclear ERs-PRs pathway that produces the modification of GnRH mRNA synthesis, as found in the NPS and OVX *in vivo* experiments. The rise in GnRH mRNA expression and total protein release induced by P4, with low frequency of GnRH release, would confirm the different actions of E2, with a) genomic effects through ER-PR acting as transcription factors at the GnRH pre-ovulatory surge, and b) rapid effects over the frequency of GnRH release that selects between the expression of LH or FSH. In addition, the present results provide the opportunity to design future studies to elucidate the modulation of rapid changes of GnRH delivery frequency which plays a key role in the reactivation of the reproductive axis during the pregnancy of this species.

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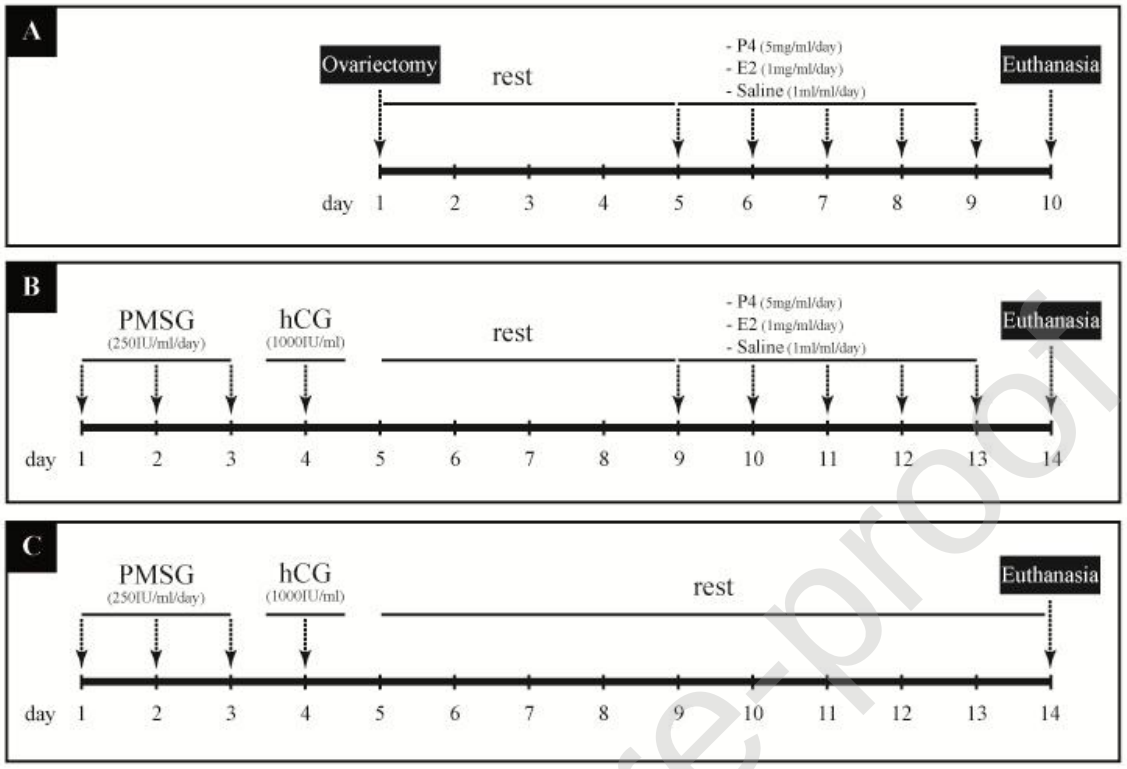

Figure 1. Layout of experimental treatments. (A) *Experiment 1:* non-pregnant females were bilaterally ovariectomized and treated, from the fifth day after surgery, by daily intramuscular administration of pharmacological doses of estradiol (E2) (1mg/kg/day), progesterone (P4) (5mg/kg/day) or vehicle during five consecutive days. Females were sacrificed the day after the last injection. **(B)** *Experiment 2:* non-pregnant females were injected intramuscularly with pregnant mare's serum gonadotropin (PMSG) (250IU/day) during three consecutive days, followed by an intramuscular administration of human chorionic gonadotropin (hCG) (1000IU) 24 hours later. After a five-day resting period, females were treated daily with pharmacological doses of E2 (1mg/kg/day), or P4 (5mg/kg/day) or vehicle during five consecutive days. Females were sacrificed fourteen days after the first injection of PMSG. **(C)** *Experiment 3:* non-pregnant females were injected intramuscularly with PMSG (250IU/day) during three consecutive days, followed by an intramuscular administration of hCG (1000IU) 24 hours later, and left to rest for ten consecutive days before euthanasia.

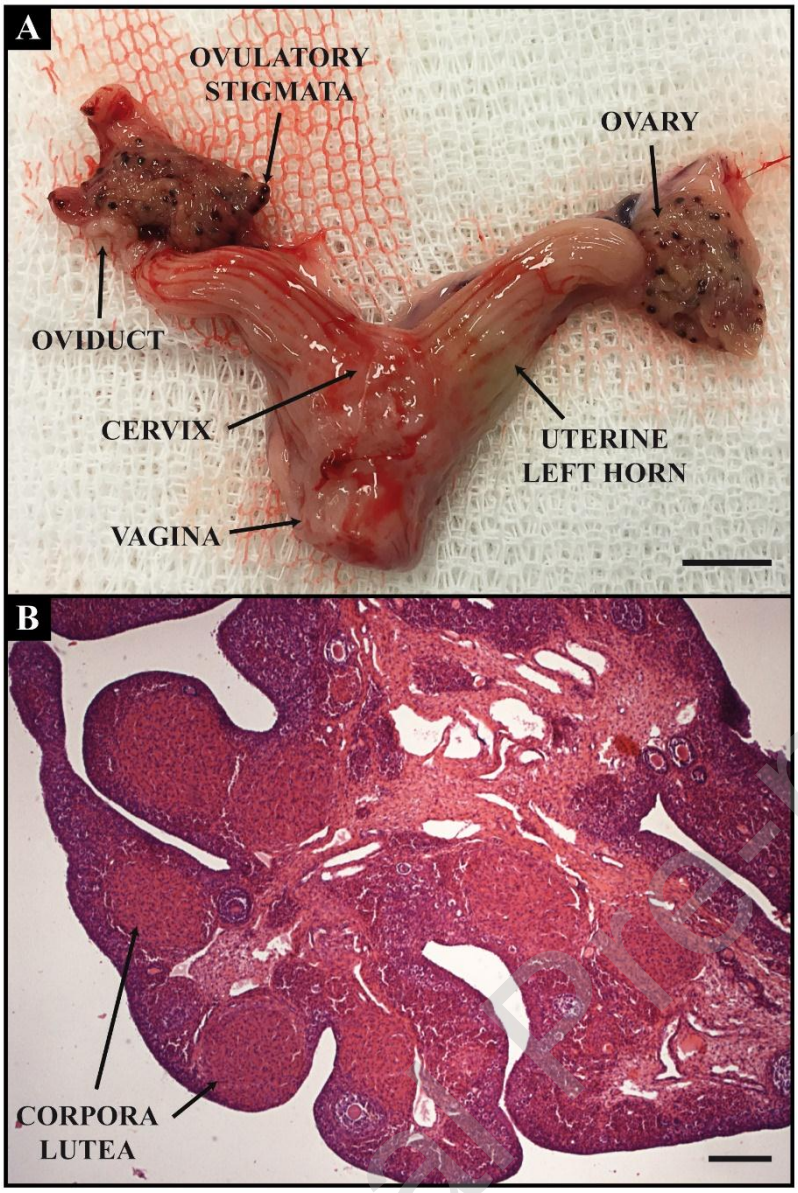

Figure 2. Luteal phase ovary. (A) Representative macroscopic image of the reproductive tract of a synchronized non-pregnant (SNP) female. Ovulatory stigmata scattered throughout the surface of both ovaries can be observed. **(B)** Representative histological image of an ovary of a SNP female with high abundance of corpora lutea; stained by Hematoxilin & Eosin. Scale bars: A) 1cm; B) 200µm.

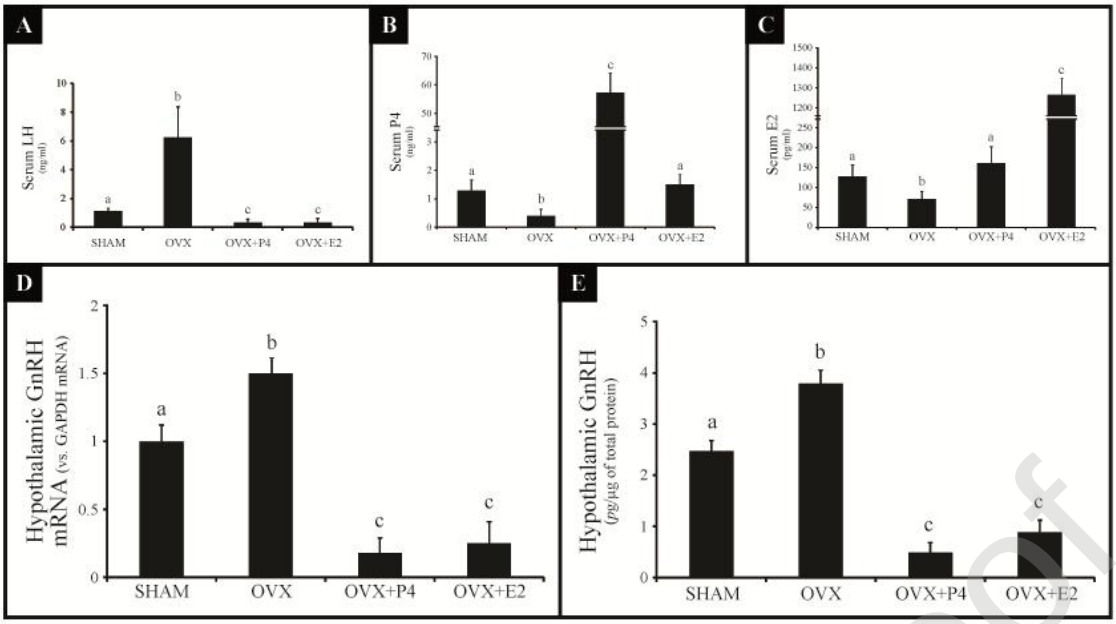

Figure 3. Serum and hypothalamic hormone levels in vizcachas with activation of hypothalamic-pituitary axis. (A) LH serum levels; **(B)** progesterone (P4) serum levels; **(C)** estradiol (E2) serum levels; **(D)** hypothalamic GnRH mRNA levels; and **(E)** hypothalamic GnRH protein content levels. Different letters indicate significant differences among groups with $p<0.05$. Data are plotted as mean ± SD. Five animals were evaluated per group. SHAM: surgery simulated non-pregnant females; OVX: ovariectomized non-pregnant females; OVX+P4: ovariectomized non-pregnant females treated *in vivo* with pharmacological doses of progesterone; OVX+E2: ovariectomized non-pregnant females treated *in vivo* with pharmacological doses of estradiol.

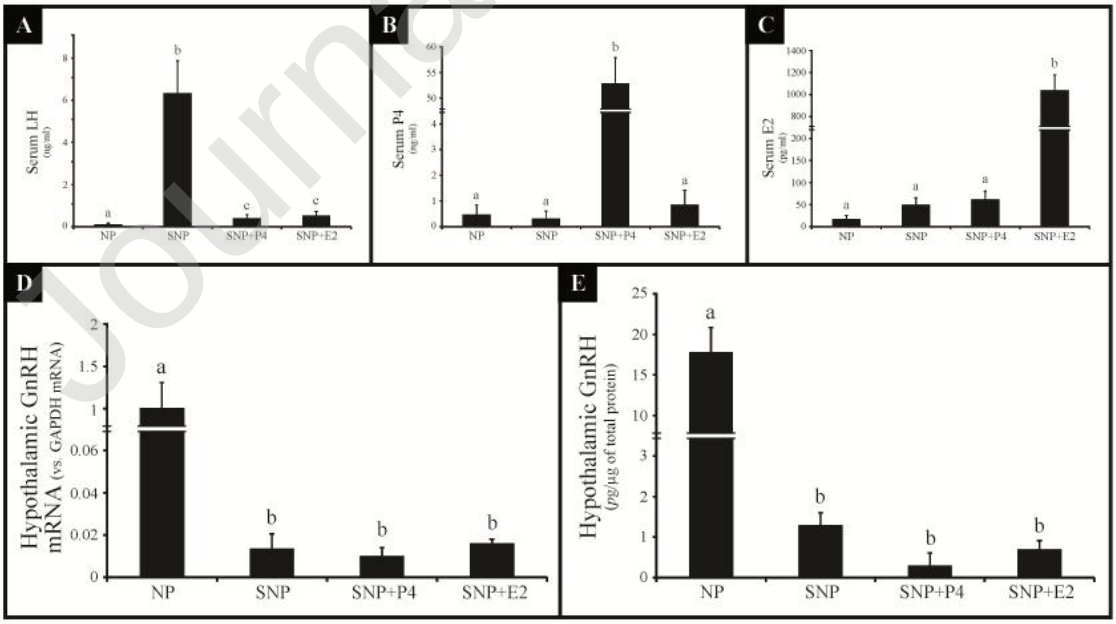

Figure 4. Serum and hypothalamic hormone levels in vizcachas during induced luteal phase. (A) LH serum levels; **(B)** progesterone (P4) serum levels; **(C)** estradiol (E2) serum levels; **(D)** hypothalamic GnRH mRNA levels; and **(E)** hypothalamic GnRH protein content levels. Different letters indicate significant differences among groups with $p<0.05$. Data are plotted as mean±SD. Five animals were evaluated per group. NP: non-pregnant females; SNP: synchronized non-pregnant females; SNP+P4: synchronized non-pregnant females treated *in vivo* with pharmacological doses of progesterone; SNP+E2: synchronized non-pregnant females treated *in vivo* with pharmacological doses of estradiol.

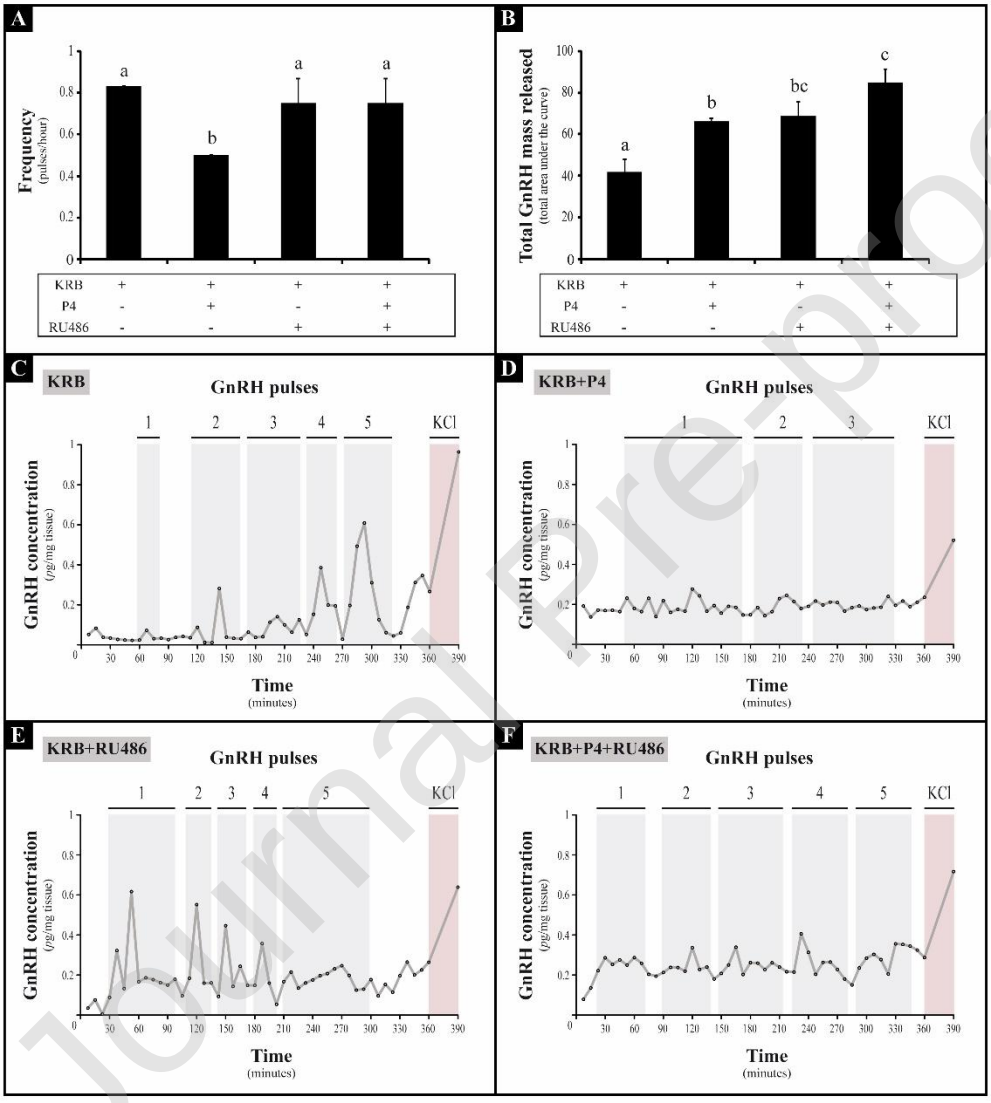

Figure 5. Hypothalamic progesterone effect on GnRH pulsatile release in vizcachas during induced luteal phase. (A) GnRH pulsatile release frequency; **(B)** GnRH total mass released during the 6-hour experiment; representative graphs of GnRH secretion (pg) in **(C)** control hypothalami (KRB); **(D)** hypothalami treated *ex vivo* with progesterone (P4); **(E)** hypothalami treated *ex vivo* with progesterone receptors antagonist (RU486); and **(F)** hypothalami treated *ex*

vivo with P4 and RU486. GnRH pulses are shadowed in grey. Final GnRH peak induced by KCl is shadowed in red. Different letters indicate significant differences among groups with $p<0.05$. Data are plotted as mean ± SD. Five animals were evaluated per group. KRB: Krebs-Ringer buffer.

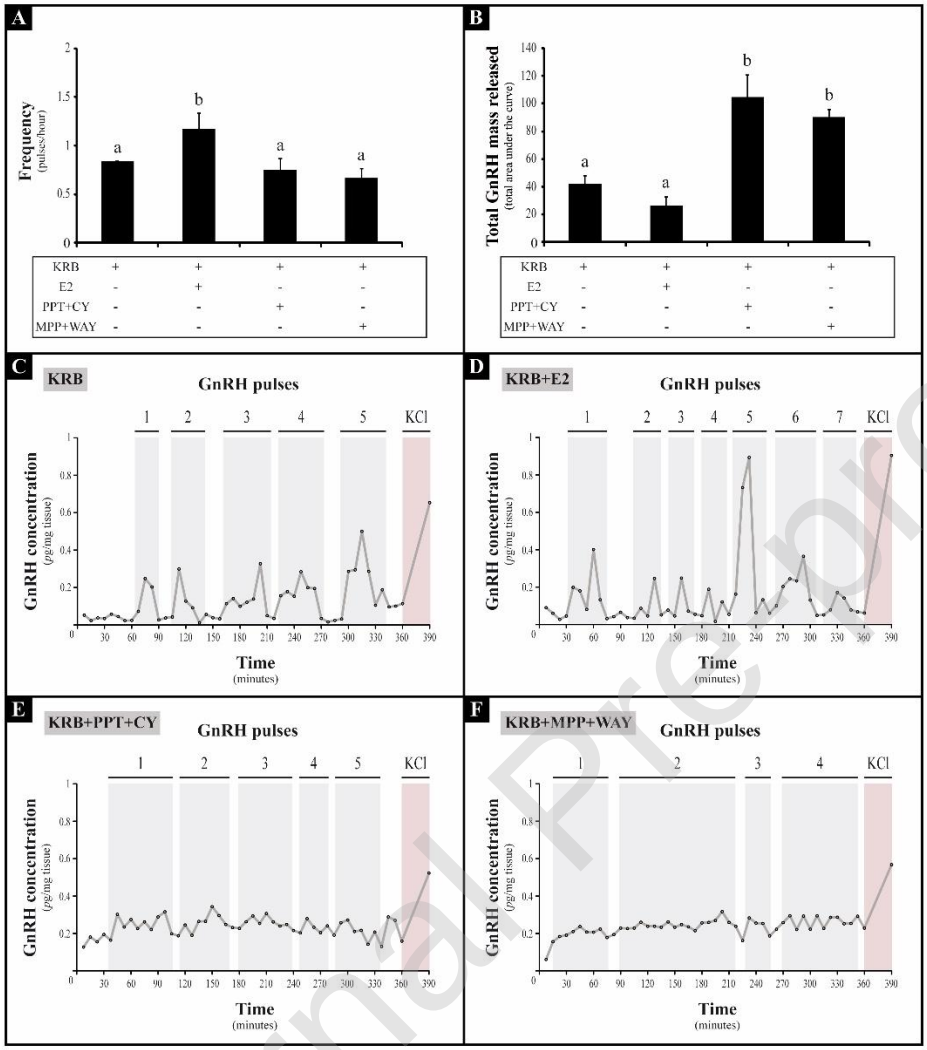

Figure 6. Hypothalamic estrogen effect on GnRH pulsatile release in vizcachas during induced luteal phase. (A) GnRH pulsatile release frequency; **(B)** GnRH total mass released during the 6-hour experiment; representative graphs of GnRH secretion (pg) in **(C)** control hypothalami (KRB); **(D)** hypothalami treated *ex vivo* with estradiol (E2); **(E)** hypothalami treated *ex vivo* with an estrogen receptor α (ERα) agonist (PPT) and with an estrogen receptor β (ERβ) antagonist (CY); and **(F)** hypothalami treated *ex vivo* with an estrogen receptor α (ERα) antagonist (MPP) and with an estrogen receptor β (ERβ) agonist (WAY). GnRH pulses are shadowed in grey. Final GnRH peak induced by KCl is shadowed in red. Different letters indicate significant differences among groups with $p<0.05$. Data are plotted as mean ± SD. Five animals were evaluated per group. KRB: Krebs-Ringer buffer.

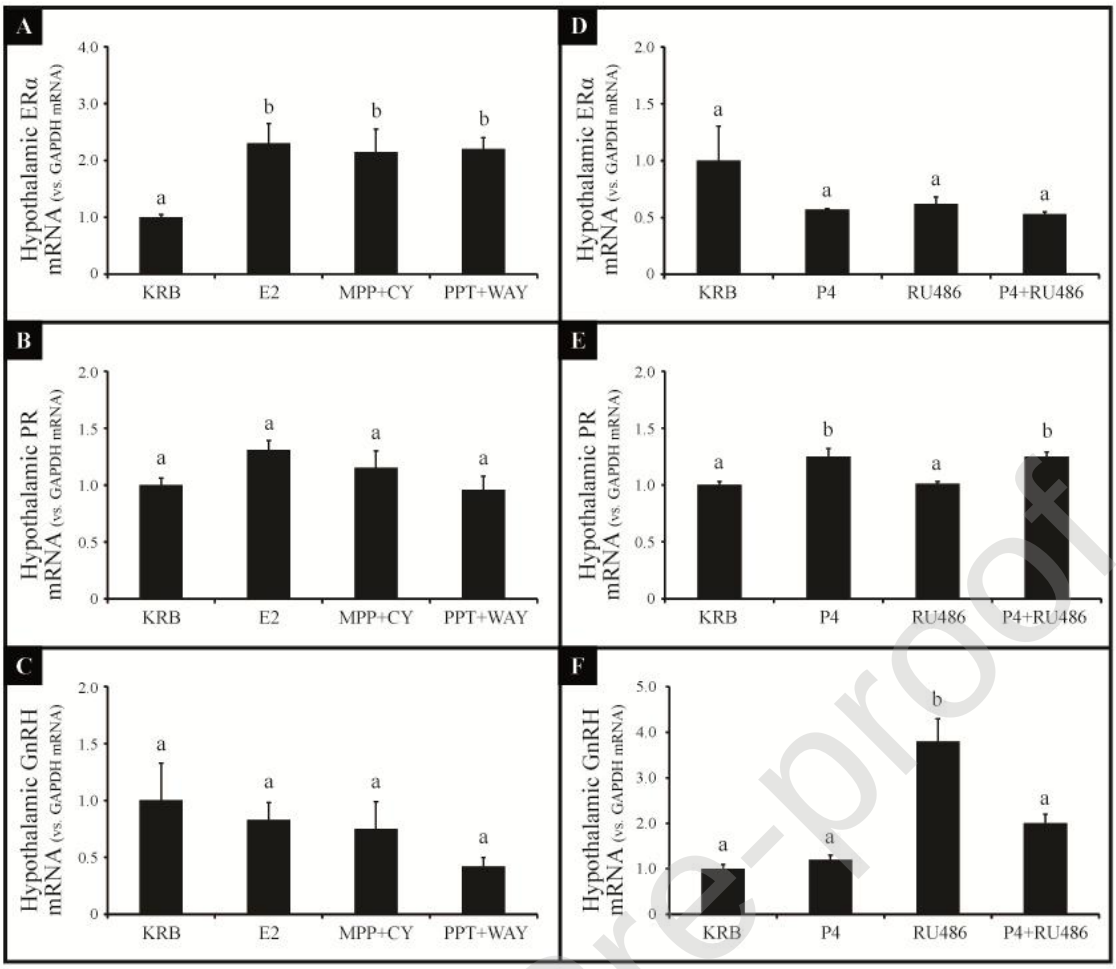

Figure 7. Hypothalamic genomic effect of progesterone and estrogen in vizcachas during induced luteal phase. Relative expression of **(A & D)** estrogen receptor α (ERα); **(B & E)** progesterone receptor (PR); and **(C & F)** GnRH mRNA levels determined by quantitative PCR (qPCR). **(A-C)** Hypothalami treated *ex vivo* with estradiol (E2), or with an estrogen receptor α (ERα) agonist (PPT) and an estrogen receptor β (ERβ) antagonist (CY), or with an estrogen receptor α (ERα) antagonist (MPP) and an estrogen receptor β (ERβ) agonist (WAY), respectively. **(D-F)** Hypothalami treated *ex vivo* with progesterone (P4), or with a progesterone receptor antagonist (RU486), or with P4 and RU486, respectively. Different letters indicate significant differences among groups with $p < 0.05$. Data are plotted as mean ± SD. Five animals were evaluated per group. KRB: Krebs Ringer buffer; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Table 1. Experimental treatments for the analysis of *ex vivo* GnRH release.

Group	Treatment	Product data
P4	- Progesterone (P4, 1μM)	- Sigma-Aldrich S.A. (N° P8783)
RU486	- Mifepristone (RU486, 10μM) - specific progesterone receptor antagonist	- Sigma-Aldrich S.A. (N° M8046)
P4+RU486	- P4 (1μM) - RU486 (1μM)	-----
E2	- 17β-estradiol (E2, 1nM)	- Sigma-Aldrich S.A. (N° E2758)
PPT+CY	- 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT, 10μM) - specific estrogen receptor α agonist - Cyclofenil (CY, 10μM) - specific estrogen receptor β antagonist	- Sigma-Aldrich S.A. (N° H6036) - Sigma-Aldrich S.A. (N° C3490)
MPP+WAY	- Methyl-piperidino-pyrazole hydrate (MPP, 10μM) - specific estrogen receptor α antagonist - WAY-200070 (WAY, 10μM) - specific estrogen receptor β agonist	- Sigma-Aldrich S.A. (N° M7068) - Sigma-Aldrich S.A. (N° W1520)

Table 2. Primers and quantitative PCR cycling parameters.

Target	Primer sequence (5'-3')	Cycle conditions	Amplicon sequence (5'-3')	Length	Homology
GnRH	F: CAGCACTGGTCCTATGGGTTGCG R: TTCCTCTTCAATCAGACGTTCC	- 1 cycle (10 min 95 °C) - 40 cycles (1	CAGCACTGGTCCTATGGGTTGCGTCCTGGAGGAAAGAGAAATGCTGAAACGTGGTTGATTCTTTCCAAGAGACAGCCAAGGAGATGAATCAACTGGCAGAACCCCAGCACTTCGAATGCACCCTCCACCAGCCTCGCTCTCCCCTCAGGGACCTGAGAGGTGTTCTGGAACGTCTGATTGAAGAGGAA	189 pb	*Mus musculus* (93%) *Homo sapi*

		5sec 95 °C; 30sec 60 °C; 30sec 72 °C)			*ens*: (93 %)
PR	F: AAGCCAGCCAGAGCCCACAR R: TGCTGCCCTTCCATYGCCC	- 1 cycle (10 min 95 °C) - 40 cycles (15sec 95 °C; 30sec 60 °C; 30sec 72 °C	GCTCGAGATCCTGTCTTATCTGTGGGGATGAAGCATCAGGCTGTCACTACGGTGTCCTTACCTGTGGGAGCTGTAAGGTCTTCTTTAAGAGGGCAATGGAAGGGCAGCAA	148 pb	*Chinchilla lanigera* (96 %) *Homo sapiens* (96 %)

		)			
ERα	F: CCTCCCGCCTTCTACAGGT R: CACACGGCACAGTAGCGAG	- 1 cycle (10 min 95 °C) - 45 cycles (15sec 95 °C; 30sec 55 °C; 30sec 60 °C; 30sec 72 °C)	TCGTCCGCCTCGCAGGTCTCGCCCAGCATCAACAGAGTAGGCAAGCATGACCCTGGAATCTGCCAAGCAGTCTCGCTACTGTGCCGTGTGAG	128 pb	*Mus musculus* (64 %) *Cameleus ferus* (88 %) *Chinchilla lanigera* (88 %)
GAPDH	F: CCAGAACATCATCCCTGCAT R: GTTCAGCTCT	- 1 cycle (10 min 95	CCAGAACATCATCCCTGCATCCACCGGTGCTGCCAAGGCTGNTGGGCAAGGTCATCCCAGAGCTGAAC	67 pb	*Mus musculus* (97

	GGGATGACCTT	°C) - 40 cycles (15sec 95 °C; 30sec 60 °C; 30sec 72 °C)			%) *Chinchilla lanigera* (92 %) *Homo sapiens*: (91 %)

F: forward; **R:** reverse; **min:** minutes; **sec:** seconds. Primers were previously employed in vizcacha.