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Chronic low BPS exposure through diet impairs *in vitro* embryo production parameters according to metabolic status in the ewe

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ABSTRACT

Bisphenol A (BPA), an endocrine disruptor, has been replaced by structural analogues including bisphenol S (BPS). BPA and BPS exhibited similar effects regarding reproductive functions. Moreover, metabolic status and lipid metabolism are related to female fertility and could worsen BPS effects. The objective was to determine BPS *in vivo* effects on folliculogenesis and embryo production after chronic exposure through diet, and the influence of metabolic status in adult ewes. Sixty primiparous 2.5 year-old ewes, undergoing a restricted or well fed diet, were exposed to BPS (0, 4 or 50 μg/kg/day) for at least three months. After hormonal oestrus synchronisation and ovarian stimulation, ewes were subjected to ovum pick-up (OPU) procedures to collect immature oocytes, that underwent *in vitro* maturation, fertilisation and embryo production. Body weight, body condition score and plasma glucose were higher in well-fed compared to restricted ewes, while plasma NEFA was lower during the 4–5 months after the beginning of the diets. Plasma progesterone levels increased on day 5 before OPU session in well-fed compared to restricted ewes. No effect of BPS dose was observed on follicle population, plasma AMH levels and embryo production numbers and rates. However, a significant diet x BPS dose interaction was reported for cleaved embryos, > 4-cell embryos, blastocyst and early blastocyst numbers, and plasma triiodothyronine levels. Our study showed that a contrasted diet did not affect follicle population nor embryo production in adult ewes but could affect the quality and progesterone secretion of the corpus luteum. Chronic low BPS exposure had no effect on follicular population and oocyte competence. Nevertheless, the significant diet x dose interactions observed on embryo production suggest that BPS effect is modulated by metabolic status. Further studies are required to assess the risk of BPS exposure for public reproductive health.

1. Introduction

Bisphenols, especially bisphenol A (BPA), are widespread compounds used in the plastic industry to produce food packaging, metal cans and cash receipt coatings, adhesive plastics, medical devices, dental sealants and cosmetics, including lacquers, varnishes, and liners (Chen et al., 2016). BPA was the first and most commonly used bisphenol and is therefore now ubiquitously present in the environment. Indeed, BPA is detected in soil, air, house dust, and in food and water (Hao, 2020). Diet is the main route of exposure for human population (Vandenberg et al., 2007) because bisphenols can transfer from packaging to content and therefore contaminate food. BPA is an endocrine disruptor (Rochester, 2013) and it exerts weak oestrogenic activity by binding to the oestrogen receptors ERα and ERβ with low affinity (Grignard et al., 2012).

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Regarding female reproduction, high urinary BPA concentrations were associated with reduced plasma oestradiol, fewer antral follicles, and a decreased number and quality of oocytes in women undergoing IVF protocols (Ehrlich et al., 2012). BPA was also reported to disturb human and bovine folliculogenesis, oocyte maturation and blastocyst formation *in vitro* (Machtinger and Orvieto, 2014). Besides, numerous studies have shown that BPA affects steroid production in rat, ovine, porcine, and human granulosa cells (Teteau et al., 2020).

In response to BPA regulation in Europe and prohibition in food contact plastic production in some countries such as France (European-Food-Safety-Authority, 2015), manufacturers started to use BPA structural analogues (Chen et al., 2016), including bisphenol S (BPS) (Thoene et al., 2020). BPS is therefore now detected in the environment and in urine at the same concentration range as BPA (0.02–21 ng/mL or 0.09–91 nM (Liao et al., 2012)). A few studies have already shown that BPS exerts endocrine disrupting effects (Eladak et al., 2015) and impairs reproduction in several species. For example, in fish and rodent species, BPS affects germinal cells and endocrine function similarly to BPA (Ullah et al., 2016). In Zebrafish, 75 days of BPS exposure is reported to increase plasma oestradiol levels and decrease egg production and sperm count (Naderi et al., 2014). In female mammals, BPS disrupts the oestrous cycle, folliculogenesis and early developmental oocyte competence. Indeed, in rats, neonatal exposure to BPA or BPS for 10 days delays puberty onset and oestrous cycle (Ahsan et al., 2018). In mice, chronic exposure of BPS for 4 weeks decreased ovary weight and the number of primary, preantral and antral follicles (Nevoral et al., 2018). Such a prolonged exposure increases spindle malformation and chromosome misalignment in mice oocytes and reduces embryo cleavage rate (Nevoral et al., 2018) and blastocyst rate (Nourian et al., 2017). Some studies showed that BPS exposure during *in vitro* maturation alters metaphase II achievement in porcine oocyte (Zalmanova et al., 2017) and damages spindle morphology and chromosome alignment in matured bovine oocytes (Campen et al., 2018a). Recently, we reported that BPS and BPA disrupted *in vitro* steroidogenesis in ovine granulosa cells (Teteau et al., 2020). BPS inhibits progesterone secretion in human and ovine granulosa cells (Amar et al., 2020; Teteau et al., 2020). BPS also inhibits oestradiol secretion in human and porcine granulosa cells (Amar et al., 2020; Bujnakova Mlynarcikova and Scsukova, 2021), while it increased basal oestradiol secretion in ovine and bovine granulosa cells (Campen et al., 2018b; Teteau et al., 2020). BPS also negatively affects ewe oocyte quality *in vitro*, even at nanomolar concentrations (Desmarchais et al., 2020). Moreover, BPA was also shown to affect cumulus cell viability (Mansur et al., 2017).

In addition to endocrine disruptors, ovarian cells are also sensitive to metabolism and especially lipid metabolism. Indeed, in females, reproduction and metabolism are tightly connected and reciprocally regulated (Fontana and Torre, 2016). Body weight, body composition, physical activity, and diet are all factors that can influence female fertility (Chavarro et al., 2007). Overweight or underweight women have the same risk of infertility and ovulatory disorders (Chavarro et al., 2007). More specifically, lipid metabolism is essential for ovarian function and supports oocyte quality (Dunning et al., 2014). Nevertheless, a high level of lipids is detrimental for oocyte quality (Leroy et al., 2014). Interestingly, cumulus cells can reduce lipid toxicity toward the oocyte by internalising follicular fluid lipids and desaturating fatty acids in order to reduce their toxicity and to store them as triglycerides in lipid droplets (Aardema et al., 2017). Nevertheless, this mechanism of oocyte protection impairs cumulus cell viability (Marei et al., 2017). In addition, as it is the case with BPA, BPS could influence energy metabolism factors such as glucose and insulin and consequently affect oocyte quality through these mechanisms (Rancière et al., 2019). Nevertheless, endocrine disruptor effects are rarely studied according to the metabolic status.

As cumulus cells could exert a protective role on cumulus oocyte complexes faced with environmental contaminants (Campen et al., 2017), we thus hypothesised that BPS could impact oocyte quality after chronic exposure and that the sensitivity of ovarian cells could vary according to metabolic status. Indeed, in cases of "intense" metabolism, CC viability might already be affected and could therefore render the oocyte more sensitive to the effects of BPS. It is thus necessary to assess the effects of oral BPS exposure in a monoovulating mammalian species like the ewe, as it is a relevant model for bisphenol toxicological studies (Gingrich et al., 2019) and for human reproductive physiology (Monniaux et al., 2014). The aims of the present study were to evaluate the influence of BPS exposure at low doses, through diet, on the quantity and size of follicles available for aspiration in the ovum pick-up (OPU) procedure (current technique used for human assisted reproduction) and on the competence of these OPU-derived oocytes subjected to *in vitro* maturation, fertilisation and the production of embryos in the ewe, regarding metabolic status. The novelty of the present study is that metabolic status is questioned as a factor that could affect individual sensitivity to BPS effects at the ovarian level.

2. Methods

2.1. Ethics

All experimental procedures were conducted in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes and approved by the French Ministry of National Education, Higher Education, Research and Innovation after ethical assessment by the local ethics committee "Comité d'Ethique en Expérimentation Animale Val de Loire (CEEA VdL)" (protocols registered under APAFIS numbers 13965–2018042008519239v2 and 14014–2018030717477406v2).

2.1.1. Chemicals

All chemicals used for IVP were purchased from Sigma Aldrich (Madrid, Spain) unless otherwise specified. All FSH and LH used in this study was highly purified porcine FSH and LH provided by J.F. Beckers (Stimufol®, Reprobiol, Liege, Belgium). Quantities are expressed in mg of the NIH-FSH-P1 standard (1 mg NIH-FSH-P1 = 0.038 mg NIADDK-oFSH-17).

2.1.2. Experimental design

A total of 60 primiparous (mean of 2.5 years old) Ile-de-France ewes were managed in a loose sheepfold at the INRAE Experimental Unit PAO (Nouzilly, France). Each pen of 21 m^2 housed ten animals as a single experimental group, and the animals were free to interact with each other. Contacts with rams and bucks or their odours were avoided during the study, and social interactions among ewes were limited to ewes of the same treatment group. Feed was distributed once a day, in the morning, and ewes had free access to water and mineral licks to provide essential micronutrients.

Before the beginning of the experiment, the ewes were assigned to two diet groups named Restricted (R, n = 30) and Well-fed (WF, n = 30) groups (diet management is explained in the following section). Animals were distributed homogeneously between the 6 experimental groups according to their age and body weight. Specific diet and BPS treatment of each group started in June. Among the R and WF groups, ewes were supplemented with or without BPS (0, 4 or 50 μg/kg/day), therefore generating 6 experimental groups named R-0, R-4, R-50, WF-0, WF-4, and WF-50 (Supplementary Table 1). As BPS is not regulated so far, these doses were based on current European BPA tolerable daily intake (TDI) of 50 μg/kg/day and temporary TDI (t-TDI) of 4 μg/kg/day, established by the European food safety agency respectively in 2006 and 2015 (European-Food-Safety-Authority, 2015).

BPS was added to the diet and adjusted once a month according to the mean LW of the ewes. After at least 3 months of treatment, all 60 ewes underwent two OPU sessions conducted at 7-day interval during the breeding season (between September and December). For technical feasibility, the number of OPU performed in one day was limited to 10,

therefore the 60 ewes were distributed into 6 batches (B1 to B6) of 10 ewes, so that all 6 experimental groups were included into each batch (Fig. 1). Recovered cumulus–oocyte complexes (COCs) were then subjected to *in vitro* maturation, fertilisation and embryo culture procedures. Ewes were sacrificed after oestrus synchronisation (see below), without ovarian stimulation, 3 weeks after the second OPU session. Preovulatory follicular fluid samples were also collected from the 60 ewes to enable progesterone and oestradiol assays. Therefore, body weight, body condition score, plasma glucose, plasma NEFA, plasma BPS-g, plasma BPS, plasma progesterone and oestradiol, pre- ovulatory follicle progesterone and oestradiol, plasma thyroid hormones and embryo production data were monitored on these 60 ewes. To provide more data on BPS exposure, urine was collected during the sacrifice of 6 ewes to enable a BPS and BPS glucuronide assay in urine (1 R-0, 2 WF-0, 2 R-50, 1 WF-50). BPS was also measured in follicular fluid collected from independent animals undergoing the exact same diet management and BPS exposure. Therefore, on these 26 additional ewes, only BPS and BPS glucuronide assays were performed.

2.1.3. Diets and nutritional management

Feed was distributed once a day, in the morning, and ewes had free access to water and mineral licks to provide essential micronutrients. The diet consisted of straw added with dietary supplement (AXEREAL Elevage, saint Germain de salles, France) composed of wheat (60%), alfalfa, sugar cane treacle and vitamins with nutritional values of 1.5 Mcal of net energy and 72 g of metabolisable protein per kilogram of dry matter and straw *ad libitum*. The diet was designed using the INRAE recommendations for the growth and maintenance needs of adult, non-pregnant ewes (Agabriel, 2013).

The ewes were weighed and their BCS recorded monthly; BCS was determined using a scale of 0 (emaciated) to 5 (grossly obese) (Russel et al., 1969). Ewes from the six experimental groups R-0, R-4, R50, WF-0, WF-4 and WF-50 (n = 10 ewes per group) were fed in order to reach the goal of a median BCS of 2.0 in R groups and 4.0 in WF groups. The quantity of the diet offered was adjusted to the mean LW of the animals in each treatment group. The ewes of the R group were first fed at 50% of their energy maintenance needs (0.15 kg of feed per animal), which corresponded to an average daily intake of 0.225 Mcal of net energy and 10.8 g of metabolisable protein. Once the target BCS was reached, the ewes were fed 80% of their energy maintenance needs (0.24 kg of feed per animal), in order to maintain the desired nutritional status. No ewes in the R groups reached one of the endpoints defined for this experiment (*i.e.*, an LW loss higher than 30% of the initial LW and/or a loss of more than 1 unit of BCS between two records) after the start of the feeding program. The ewes in the WF groups were fed at 165% of their energy maintenance needs until the end of the experiment (0.50 kg of feed per animal), which corresponded to an average daily intake of 0.75 Mcal of net energy and 36 g of metabolisable protein.

2.1.4. Hormonal treatment: oestrus synchronisation and ovarian stimulation

Each ewe underwent two OPU sessions 7 days apart. Prior the first OPU session, all ewes received two intramuscular injections of 125 μg/ewe cloprostenol (Estrumate®, MSD Santé Animale, Beaucouzé, France) 8 days apart, and 5 days after the second injection, a 30–mg fluorogestone acetate (FGA) vaginal sponge (SYNCHRO-PART® 30 mg, CEVA Santé Animale, Libourne, France) was inserted for 12 days, until the first OPU session. This FGA sponge was renewed on the day of the first OPU session to prepare the ewes to the second one 7 days later. Ovarian stimulation was performed under FGA treatment. For ovarian stimulation, the 60 ewes underwent the same protocol by receiving a total of 32 mg FSH (Stimufol®, Reprobiol, Liège, Belgium) in five intramuscular injections in decreasing doses: 8 mg (60 h), 8 mg (48 h), 6 mg (36 h), 6 mg (24 h) and 4 mg (12 h) during the three days preceding the first

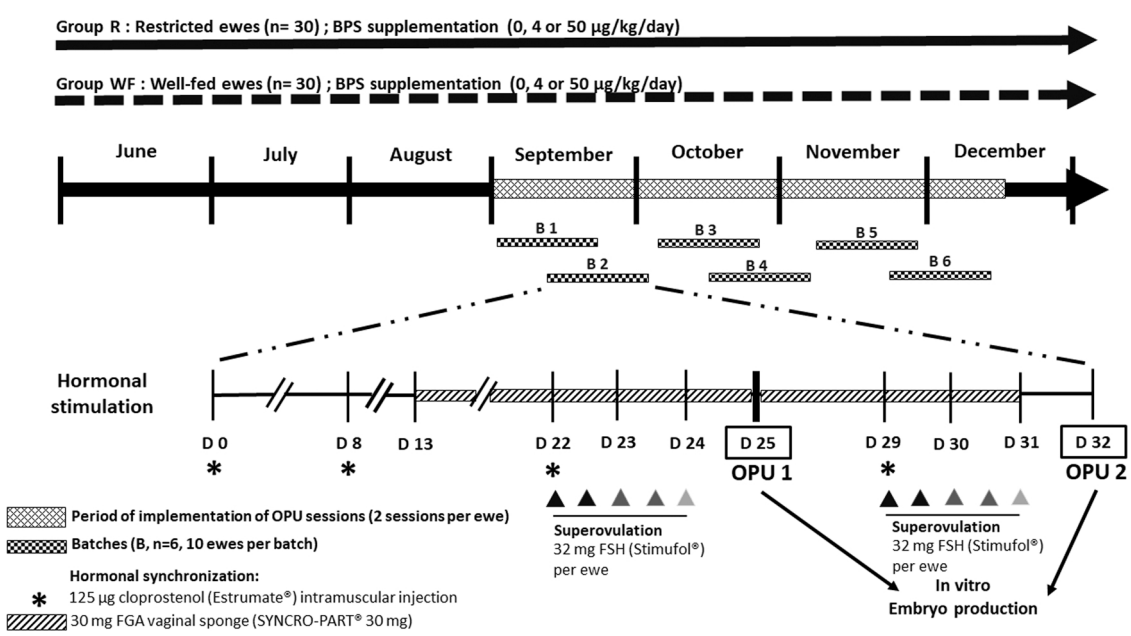

Fig. 1. Experimental design. Ewes (n = 60) were divided into six groups: restricted without BPS (R-0, n = 10), restricted with BPS 4 μg/kg/day (R-4, n = 10), restricted with BPS 50 μg/kg/day (R-50, n = 10), well-fed without BPS (WF-0, n = 10), well-fed with BPS 4 μg/kg/day (WF-4, n = 10), well-fed with BPS 50 μg/kg/day (WF-50, n = 10). The specific diets and BPS treatments were maintained during the whole experimental period. After at least 3 months of treatment, ewes underwent two follicular puncture (OPU) sessions 7 days apart, following estrus synchronization and ovarian stimulation. For technical feasibility (10 OPU per day), the ewes were distributed into 6 batches (B1 to B6), so that all 6 groups were included into each batch. OPU sessions of each batch started with estrus synchronization of the ewes by administration of 125 μg cloprostenol (Estrumate®) on day 0 (D0) and D8. Five days later (D13), ewes received a 30 mg fluorogestone acetate (FGA) vaginal sponge (Syncro part PMSG®). A new cloprostenol injection was performed the 1st day of ovarian stimulation (D22). For ovarian stimulation treatment, ewes received 32 mg FSH (Stimufol®) in 5 injections in decreasing concentrations during the 3 days before OPU at D25. FGA treatment was renewed the day of the 1st OPU session, and ovarian stimulation started again 4 days later to perform OPU 2. Recovered cumulus–oocyte complexes (COCs) were subjected to *in vitro* maturation, fertilization and culture.

and the second OPU session (Lahoz et al., 2014). Coinciding with the first injection of FSH, 125 μg/ewe cloprostenol was administered.

2.1.5. Recovery of oocytes by OPU

Donor ewes were fasted for 12 h prior to each OPU session. Ewes were anaesthetised intravenously (i.v.) with 0.05 mg/kg xylazine (ROMPUN® 2%, Bayer Division Animal Health, La Garenne-Colombes, France) and 2–4 mg/kg ketamine (IMALGENE®, Merial, Lyon, France) followed by inhalation maintenance anaesthesia with 2,5% isofluorane (Piramal, India). Follicular puncture was performed by a modified procedure based on the technique previously described by Alberio et al. (2002). An endoscope was inserted through a 1 cm abdominal trocar incision, and atraumatic grasping forceps were introduced through a contralateral incision to immobilise the ovary. By a third 1 cm incision, an Ova-Stiff™ EchoTip® needle (Cook medical, Ireland) connected to a vacuum pump adjusted to 100 mmHg was introduced to aspirate all visible ovarian follicles ≥ 2 mm in diameter. Cumulus oocyte complexes (COC) from both ovaries were collected individually for each ewe, into 15 mL falcon tube containing phosphate buffer saline (PBS) at 38.5 °C. During the surgery, the number and diameter of punctured follicles were recorded for each ewe and OPU session, using a 2 mm scale positioned on the needle. After intervention, warm physiological saline (0.9% NaCl) has been injected into the abdominal cavity and the trocar holes were closed with surgical staples. An injection of long-acting non-steroidal anti-inflammatory drug was injected intramuscularly (2 mg/kg of flunixine meglumin, Finadyne®, MSD Santé Animale, Beaucouzé, France) at the end of the procedure.

2.1.6. In vitro maturation (IVM), in vitro fertilisation (IVF) and in vitro embryo development

COC recovered from both OPU sessions underwent *in vitro* maturation in 4-well dishes (Nunclon surface treated, Nunc, ThermoFischer Scientific, Illkirch, France) individually for each ewe, with up to 13 COCs per 100 μL of maturation medium composed as previously described (Desmarchais et al., 2020) and covered with mineral oil. When 14 or more COCs were recovered, they were divided in several drops of medium in ≤ 13 COC groups. IVM was performed for 24 h at 38.8 °C in a humidified atmosphere containing 5% CO_2. Matured COCs were transferred for washing in IVF medium and underwent fertilization (IVF) with 3×10^6 spermatozoa/mL from a semen pool from five ram ejaculates as previously described (Desmarchais et al., 2020).

Spermatozoa and COCs, gathered individually for each ewe, were coincubated for 24 h in a 100 μL modified Synthetic Oviductal Fluid medium drop (Desmarchais et al., 2020) covered with mineral oil at 38.8 °C under a humidified atmosphere of 5% CO_2 in air. Then, cumulus cells (CC) were removed, and presumptive zygotes were washed. *In vitro* embryo development (IVD) of embryos was performed in drops of modified SOF medium (1 μL/zygote) covered with mineral oil. At day 2 of IVD, 10% foetal calf serum (FCS) was added to the drop of medium. The cleavage rate and embryo development were assessed at day 2 and 6 of IVD, respectively, using a Zeiss inverted microscope (Zeiss, Germany). The cleavage rate was defined as the total number of cleaved embryos divided by the total number of fertilised COC. During embryo development analysis, the total number of blastocysts (early unexpanded blastocysts and expanded blastocysts) was recorded. All blastocyst rates were defined as the total number of blastocysts divided by the number of cleaved embryos.

2.1.7. Blood samples and assays

Blood samples (5 mL) were taken twice a month, one hour after morning feeding, by jugular venipuncture, in heparinised tubes (17 IU/mL sodium heparin, Vacutainer®; Becton Dickinson and Company, Le Pont de Claix, France). Then they were centrifuged (3700 g for 30 min at 4 °C) and plasmas were stored at-20 °C until assays were conducted. Plasma non-esterified fatty acids (NEFAs) and glucose were measured once a month during the experiment. Plasma BPS and BPS-glucuronide (BPS-g) were measured before the beginning of the exposure and on the day before the first OPU. Anti-Mullerian hormone (AMH) was measured 12 days before the first OPU. Plasma progesterone was measured 5 and 1 day before the first OPU. Plasma oestradiol and thyroid hormones were measured the day before the first OPU. Preovulatory follicle progesterone and oestradiol were measured at the end of the experiment after oestrus synchronisation, at the time of the presumptive oestrus.

2.1.7.1. Plasma non-esterified fatty acids (NEFAs) and glucose. Plasma NEFA and glucose were were quantified on a 5 and 2 μL undiluted plasma sample, respectively, by colorimetric enzymatic methods using a Konelab 20 analyzer (Thermo Scientific, Gometz le Châtel, France) and kits provided by Bio-Mérieux (Marcy-l'Etoile, France) and Thermo Scientific (Villebon sur Yvette, France) as previously described (Baéza et al., 2015).

2.1.7.2. Plasma BPS and BPS-glucuronide (BPS-g). BPS and BPS-glucuronide (BPS-g) were quantified—without resorting to a hydrolysis step—using liquid chromatography-mass spectrometry with an Acquity U-HPLC device coupled to a Xevo-TQ triple quadrupole mass spectrometer (Waters, Saint-Quentin-en-Yvelines, France) operated with positive electrospray ionisation and MRM mode. Chromatographic separation was achieved on a Waters Phenyl-Hexyl U-HPLC column (2.1 × 100 mm; 1.6 μm) with an acidified H_2O/AcN gradient elution (0.3 mL/min, 40 °C). Chromatographic data were monitored using Targetlynx® software (Waters Corporation). Briefly, the samples (250 μL) were purified with anionic exchange solid phase extraction (SPE) cartridges using BPS-g d8 (Toronto Research Chemicals) as an internal standard. The resulting extract was derivatised with chloride dansyl. All plasma were quantified within 1 day with a calibration curve that ranged from 0.05 to 200 ng/mL and 0.5–200 ng/mL for BPS-g and BPS, respectively. The limit of quantification (LOQ) was set at 0.05 ng/mL (0.10 nM) and 0.5 ng/mL (2.0 nM) for plasma BPS-g and BPS, respectively. The accuracy and precision of the assay were evaluated with two series of three quality control (QC) samples at 0.15, 0.375, 1.5, 3.75, 15 and 150 ng/mL. The mean accuracy were 99% and 101% and intra-day CV precision of the assay were 7% and 14% for plasma BPS-g and BPS, respectively. When the result of the assay was undetectable (<LOQ), the concentration was considered as 0. When the BPS concentration was positive despite an undetectable level of BPS-g, the result was considered as an environmental contamination of the sample and the data was therefore considered as lacking.

2.1.7.3. Plasma Anti-Müllerian hormone. Plasma anti-Müllerian hormone (AMH) is an ovarian response indicator after hormonal FSH stimulation. AMH was determined using the AMH Gen II ELISA assay (Beckman Coulter, Villepinte, France). Analyses were performed using 50 μL of undiluted plasma, as previously described (Rico et al., 2009), from samples collected 12 days before the 1st OPU session (5 days after second cloprostenol injection on FGA sponge insertion day).

2.1.7.4. Progesterone in plasma and follicular fluid. Plasma progesterone was determined using an ELISA assay (Canepa et al., 2008) in 10 μL of undiluted plasma samples collected 5 days (12 days after second cloprostenol injection, therefore 7 days after the treatment with the 30–mg fluorogestone acetate (FGA) vaginal sponge) and 1 day before the first OPU session. Pre-ovulatory progesterone was determined using 10 μL of undiluted follicular fluid samples collected at the time of presumptive oestrus after oestrus synchronization. The intra-day CV precision of the assay was 3.9%.

2.1.7.5. Oestradiol in plasma and follicular fluid. Plasma oestradiol was determined using an ELISA assay (DIAsource ImmunoAssays® S.A., Louvain La Neuve, Belgium, adapted for oestradiol detection in ovine plasma, as previously described (Fabre-Nys et al., 2015) in 400 μL of

undiluted plasma samples collected 1 day before the first OPU session. Pre-ovulatory oestradiol was determined using 10 μL of follicular fluid samples collected at the time of presumptive oestrus after oestrus synchronization and diluted ten-fold. The intra-day CV precision of the assay was 3.7%.

2.1.7.6. Plasma thyroid hormones. Plasma free triiodo-thyronine (FT3), free thyroxine (FT4) and total T4 (TT4) were measured on 50 μL of undiluted plasma the day before the first OPU session using the FT3, FT4 and T4 ELISA assays, respectively (Diasource, Louvain-la-Neuve, Belgium) and following the manufacturers recommandations. The intra-day CV precision of the assay was 8.5% for FT3, 5.6% for FT4 and 3.1% for TT4.

2.1.8. Statistical analyses

Statistical analyses were performed using SAS® software (SAS institute Inc, 2013).

Linear mixed models (MIXED procedure) including the effects of diet, BPS dose exposure and diet x BPS dose interaction, with batch (B1 to B6, see Fig. 1) as a random effect, were used for BW, BCS, plasma glucose and plasma NEFA measured at the time of the first OPU, plasma BPS and BPS-g, plasma AMH, plasma oestradiol, follicular fluid progesterone and oestradiol, plasma thyroid hormones.

Linear mixed models (MIXED procedure) including the effects of diet, BPS dose exposure, measurement stage and diet x BPS dose x stage interaction, with measurement stage as a repeated effect within ewe (repeated statement of the MIXED procedure, an AR(1) covariance structure was used), with ewe and batch (B1 to B6, see Fig. 1) as random effects, were used for the following parameters: BW, BCS, plasma glucose and plasma NEFA measured once a month, plasma progesterone.

Linear mixed models (MIXED procedure) including the effects of diet, BPS dose exposure and diet x BPS dose interaction, with ewe, batch and rank of OPU session as random effects, were used for the following parameters: numbers of punctured follicles, numbers of COC recovered and that underwent IVM, numbers of embryos (cleaved, >4 cells, blastocysts) produced per OPU session.

Logistic regression mixed models (GLIMMIX procedure) including the effects of diet, BPS dose exposure and diet x BPS dose interaction, with ewe, batch and rank of OPU session as random effects, were used for the following parameters: cleavage rate and embryo development rates.

Results are presented as least squares means (lsmeans) ± SEM, unless otherwise indicated. Multiple comparisons of lsmeans estimated by the models were performed using a Tukey adjustment for all parameters. Effects with $p \leq 0.05$ were considered significant, and effects with $0.05 < p \leq 0.1$ were considered tendencies.

3. Results

3.1. Body weight and body condition score

Live body weight (BW) and body condition score (BCS) were recorded once a month during the experiment, and at the time of first OPU session (Fig. 2). During the five months period following the beginning of the diet, BW and BCS averages were lower (diet effect, $p < 0.0001$) in R ewes (54.1 ± 1.2 kg and 2.18 ± 0.02, respectively) compared to WF ewes (64.3 ± 1.2 kg and 2.92 ± 0.02, respectively) (Fig. 2A. and C.). No differences were observed between BPS 0, 4 and 50 μg/kg/day dose groups (dose effect, $p = 0.42$ for BW and $p = 0.86$ for BCS). A significant effect of measurement stage ($p < 0.0001$) and of the diet x dose x measurement stage interaction ($p < 0.0001$) were observed for BW and BCS. Whatever the BPS dose group, mean BW was different between R and WF ewes (Tukey adjustment, $p \leq 0.05$) at M1 (except for dose 50), M2, M3, M4 and M5; mean BCS was different between R and WF ewes (Tukey adjustment, $p \leq 0.05$) from M0 to M5. At the first OPU session,

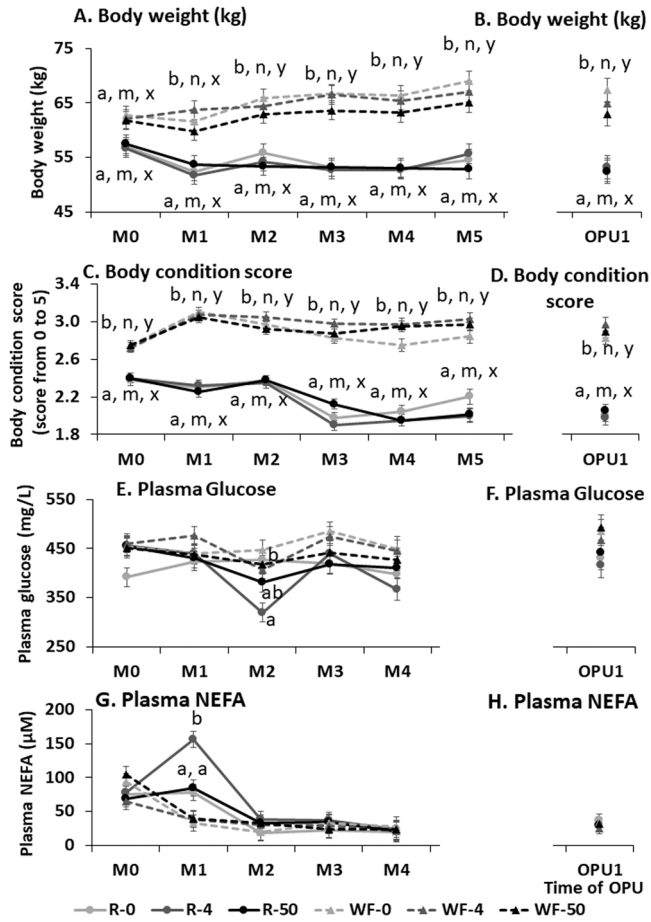

Fig. 2. Body weight (A., B.) and body condition score (C., D.), plasma glucose levels (E., F.) and plasma NEFA levels (G., H.) levels were measured once a month (M) from the beginning of the diet to the end of the experiment. BCS was determined using a scale of 0 (emaciated) to 5 (grossly obese) (Russel et al., 1969). Ewes were either restricted (R groups, n = 30, solid lines) or well-fed (WF groups, n = 30, dotted lines) and received BPS 0 μg/kg/day (n = 20, light grey lines), 4 μg/kg/day (n = 20, dark grey lines), or 50 μg/kg/day (n = 20, black lines), at least three months before the first OPU session. Due to the necessity of distributing the 60 ewes into 6 batches (for technical feasibility of the surgery and the IVF experiments), the OPU period did differ between batches in terms of duration after the beginning of the experimentation. This is the reason why the OPU period (B, D, F, H) is separated from the start of the experiment (A, C, E, G). Results are presented as lsmeans + /- SEM. (a,b), (m,n) and (x,y) indicate a significant difference ($P \leq 0.05$) between R and WF ewes at a given stage for 0, 4 or 50 μg/kg/day BPS exposure, respectively. Linear mixed models were used and multiple comparisons of lsmeans estimated by the models were performed using a Tukey adjustment for all parameters. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

BW (Fig. 2.B.) was significantly decreased (diet effect, $p < 0.001$) in R ewes (52.7 ± 1.8 kg) compared to WF ewes (65.1 ± 1.8 kg), with neither effect of BPS dose ($p = 0.21$) nor diet x BPS dose interaction ($p = 0.26$). BCS (Fig. 2.D.) was also significantly decreased (diet effect, $p < 0.0001$) in R ewes (2.01 ± 0.04) compared to WF ewes (2.90 ± 0.04), with neither effect of BPS dose ($p = 0.59$) nor diet x BPS dose interaction ($p = 0.33$).

3.1.1. Plasma glucose and NEFA levels

Metabolic measurements (glucose and NEFA) were performed in plasma once a month from the beginning of the diet, and at the time of the first OPU session. Plasma glucose levels (Fig. 2.E.) were higher (diet effect, $p < 0.0001$) in WF ewes, 447.5 ± 6.6 mg/L, compared to R ewes,

411.8 ± 6.6 mg/L. On the contrary, plasma NEFA levels (Fig. 2.G.) were significantly lower (diet effect, p = 0.048) in WF ewes, 40.9 ± 4.7 μM, compared to R ewes 52.4 ± 4.7 μM. No effect of the BPS exposure dose was reported neither for plasma glucose (p = 0.82) nor for plasma NEFA (p = 0.41). A significant effect of measurement stage (p < 0.0001) and of the diet x dose x measurement stage interaction were observed for glucose (p = 0.017) and NEFA (p < 0.0001). In R ewes, plasma glucose was different between BPS dose 0 and dose 4 at M2 following the beginning of the diet (Tukey adjustment, p = 0.031). In R ewes, plasma NEFA was different between BPS dose 0 and dose 4 (Tukey adjustment, p = 0.002) and between BPS dose 4 and dose 50 (Tukey adjustment, p = 0.007) at M1 following the beginning of the diet. Moreover, at M1, plasma NEFA at BPS dose 4 differed between R and WF ewes. At the first OPU session, plasma glucose (Fig. 2.F.) was significantly lower (diet effect, p = 0.007) in R ewes (429.3 ± 16.8 mg/L) compared to WF ewes (481.4 ± 16.8 kg), with neither effect of BPS dose (p = 0.52) nor diet x BPS dose interaction (p = 1.00). At the first OPU session, plasma NEFA (Fig. 2.H.) did not differ (diet effect, p = 0.77) between R ewes (31.9 ± 7.0 μM) and WF ewes (30.2 ± 7.0 μM), with neither effect of BPS dose (p = 0.57) nor diet x BPS dose interaction (p = 0.85).

3.1.2. *BPS and BPS-g levels in plasma, urine and follicular fluid*

As BPS-g is a marker of internal BPS chronic exposure, plasma BPS-g level was first measured before the first exposure of ewes. According to raw data, only five ewes (1 R-0, 2 R-4, 1 WF-0 and 1 WF-50 ewes), out of the 60 ewes, had plasma BPS-g levels ≥ 0.05 ng/mL (0.12 nM, LOQ of the assay) but < 3.5 ng/mL (8.2 nM), with a mean concentration of 1.16 ± 0.61 ng/mL (2.73 ± 1.43 nM). The plasma BPS-g level was also measured during the OPU period, the day before the first OPU (Fig. 3A). BPS dose (p < 0.0001), but not the diet (p = 0.39), impacted plasma BPS-g level (with no interaction between diet and dose, p = 0.73). WF-50 and R-50 ewes receiving the BPS dose of 50 μg/kg/day showed a 200–310-fold increase in plasma BPS-g compared with WF-0 and R-0 ewes, respectively (Tukey adjustment, p < 0.0001) and a 7–9-fold increase for R-50 and WF-50 compared with R-4 and WF-4 groups, respectively (Tukey adjustment, p < 0.0001). Plasma BPS-g in R-50 ewes differed from both WF-0 and WF-4 ewes, as well as plasma BPS-g in WF-50 ewes differed from both R-0 and R-4 ewes (Tukey adjustment, p < 0.0001).

Plasma BPS, indicating current exposure, was also measured the day before the first OPU (Fig. 3B). BPS dose (p < 0.0001), but not the diet (p = 0.55), impacted plasma BPS level (with no interaction between diet and dose, p = 0.46). Both R-50 and WF-50 ewes showed an increase in plasma BPS compared with R-0 and WF-0 ewes respectively (Tukey adjustment, p < 0.05). Plasma BPS was increased in R-50 ewes compared to R-4 ewes (Tukey adjustment, p < 0.05), the same tendency being observed between WF-50 and WF-4 ewes (Tukey adjustment, p = 0.089). Plasma BPS in R-50 ewes differed from both WF-0 and WF-4 ewes (Tukey adjustment, p < 0.05), and plasma BPS in WF-50 ewes differed from both R-0 and R-4 ewes (Tukey adjustment, p < 0.10).

BPS and BPS-g levels were measured in the urine 1 month after the OPU, after oestrus synchronisation, at the moment of the presumptive ovulation, for 6 ewes (among the 60 experimental ewes), exposed to either 0 (n = 3, 1 R ewe and 2 WF ewes) or 50 (n = 3, 2 R ewes and 1 WF ewe) μg/kg/day BPS. BPS averaged 0.8 ± 0.5 nM and 1.6 ± 1.3 nM in ewes exposed to 0 and 50 μg/kg/day BPS, respectively. BPS-g was not detected in ewes exposed to 0 μg/kg/day and averaged 108.6 ± 10.6 nM in ewes exposed to 50 μg/kg/day BPS. In addition, the BPS and BPS-g levels in follicular fluid from pre-ovulatory follicles were checked on 26 supplementary ewes, undergoing the same diet management and BPS supplementation as the 60 ewes of the main experiment. Pre-ovulatory follicles were punctured after 5.9 ± 0.2 months of BPS supplementation. While BPS was not detected in follicular fluid of 24 ewes out of 26, BPS-g was detected in the follicular fluid of 18 ewes (out of 26) exposed to 4 or 50 μg/kg/day BPS (supplementary Figure 1) and averaged 8.7 ± 1.1 nM in R-4 ewes (n = 4), 5.5 ± 1.3 nM in WF-4 ewes (n = 4), 102.5 ± 7.7 nM in R-50 ewes (n = 5) and 43.6 ± 20.6 nM in WF-50 ewes (n = 5).

3.1.3. *Progesterone and oestradiol levels in plasma and follicular fluid*

Plasma progesterone was measured twice: 5 days and 1 day before the first OPU session (Fig. 4.A.). Five days before the OPU session corresponded to 1 day before starting FSH follicle stimulation, and 12 days after the second injection of cloprostenol and 7 days after FGA sponge insertion (Fig. 1).

Plasma progesterone was significantly lower in R ewes compared to WF ewes (diet effect, p < 0.0001), while there was no effect of the BPS dose exposure (p = 0.25). There was a significant decrease in progesterone 1 day compared to 5 days before the OPU session (measurement stage effect, p < 0.0001), in all experimental groups. A significant effect of diet x dose x measurement stage interaction was observed (p = 0.038): five days before the OPU session, plasma progesterone was lower in R-0 compared to WF-0 ewes (Tukey adjustment, p = 0.008) and tended to be lower in R-50 compared to WF-50 ewes (Tukey adjustment,

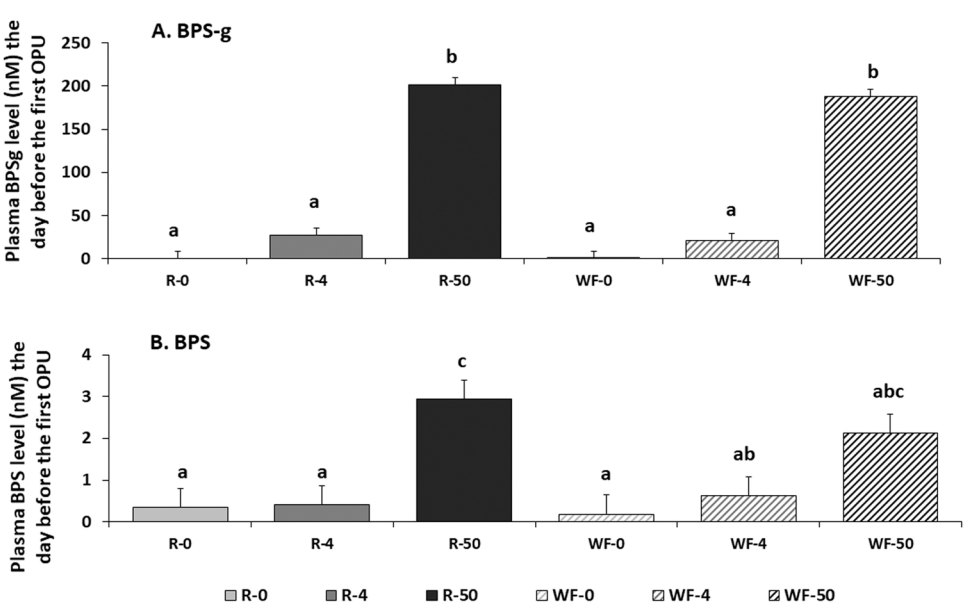

Fig. 3. Plasma BPS-g (A.) and BPS (B.) levels were measured during the OPU period, for six groups of ewes (n = 10 per group) exposed to either 0, 4 or 50 μg/kg/day BPS and undergoing a restricted (R) or a well-fed (WF) diet: R-0, R-4, R-50, WF-0, WF-4, WF50. Results are presented as lsmeans + /- SEM. Bars that do not exhibit at least one common letter are significantly different ($P \leq 0.05$). Linear mixed models were used and multiple comparisons of lsmeans estimated by the models were performed using a Tukey adjustment for all parameters.

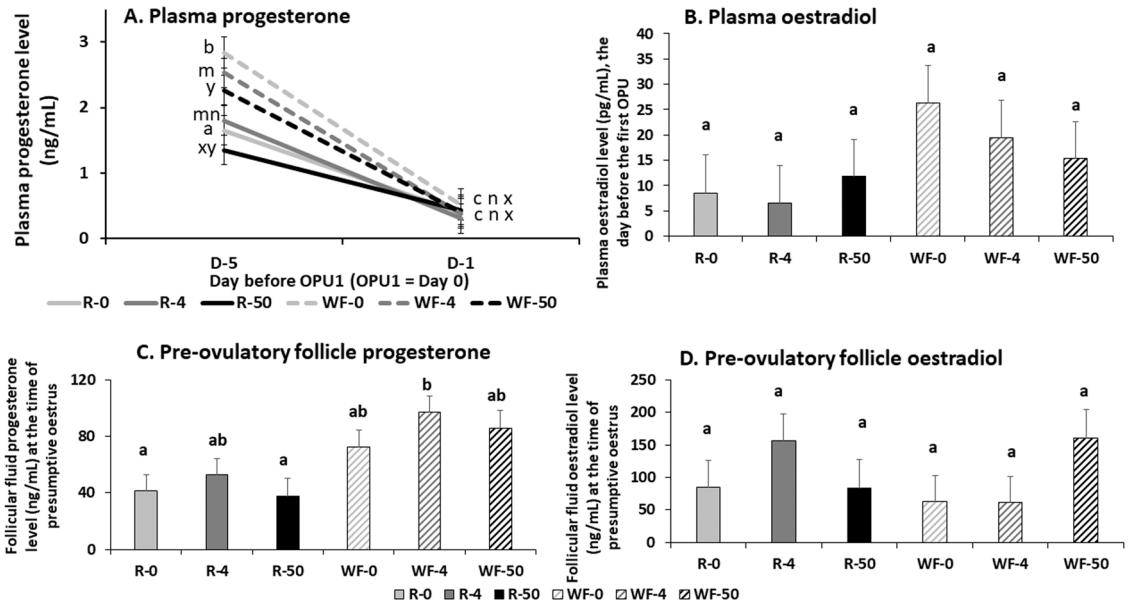

Fig. 4. Plasma progesterone levels (A.) was measured 5 days before (D-5) and the day before (D-1) the first OPU session for six groups of ewes (n = 10 per group) exposed to either 0, 4 or 50 μg/kg/day BPS and undergoing a restricted (R) or a well-fed (WF) diet: R-0, R-4, R-50, WF-0, WF-4, WF50. Plasma oestradiol (B.) was measured the day before (D-1) the first OPU session for the six groups of ewes (n = 10 per group). Follicular fluid progesterone (C.) and oestradiol (D.) were measured at the moment of ewe sacrifice for the six groups of ewes (n = 10 per group). Results are presented as lsmeans + /- SEM. (a,b,c), (m,n) and (x,y) indicate a significant difference ($P \leq 0.05$) in plasma progesterone between R and WF ewes at a given stage for 0, 4 or 50 μg/kg/day BPS exposure, respectively. Concerning plasma oestradiol, follicular fluid progesterone and oestradiol, different letters indicate a significant difference ($P \leq 0.05$). Linear mixed models were used and multiple comparisons of lsmeans estimated by the models were performed using a Tukey adjustment for all parameters.

p = 0.107). One day before the OPU session, no difference was observed between groups. Progesterone was also measured in follicular fluid from pre-ovulatory follicle, after an oestrus synchronisation, 1.12 ± 0.06 month after the first OPU (Fig. 4.C.), and was lower in R ewes compared to WF ewes (diet effect, p = 0.0001), with no effect of BPS dose (p = 0.27) or diet x dose interaction (p = 0.77). Follicular fluid progesterone was higher in WF-4 ewes compared to R-0 and R-50 ewes (Tukey adjustment, p < 0.02) and tended to be higher (p = 0.08) compared to R-4 ewes. It also tended to be higher in WF-50 compared to R-50 ewes (Tukey adjustment, p = 0.09).

Plasma oestradiol was measured 1 day before the first OPU session (Fig. 4.B.). It was significantly lower in R ewes compared to WF ewes

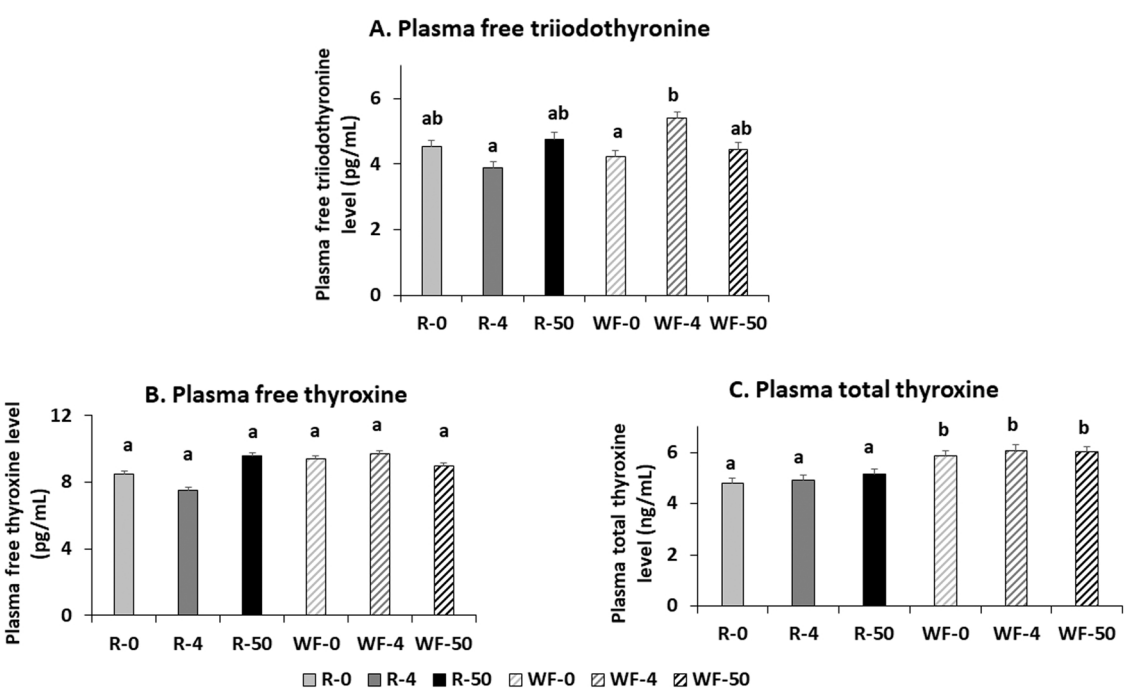

Fig. 5. Plasma free triiodothyronine (A.), free thyroxine (B.) and total thyroxine (C.) levels were measured the day before the first OPU session for six groups of ewes (n = 10 per group) exposed to either 0, 4 or 50 μg/kg/day BPS and undergoing a restricted (R) or a well-fed (WF) diet: R-0, R-4, R-50, WF-0, WF-4, WF50. Results are presented as lsmeans + /- SEM. Different letters indicate a significant difference ($P \leq 0.05$). # indicates tendency ($0.05 < P \leq 0.01$). Linear mixed models were used and multiple comparisons of lsmeans estimated by the models were performed using a Tukey adjustment for all parameters.

(diet effect, p = 0.028) with no effect of BPS dose exposure (p = 0.76) or diet x BPS dose interaction (p = 0.49). Oestradiol was also measured in follicular fluid from pre-ovulatory follicle (Fig. 4.D.): no effect of diet (p = 0.70), BPS dose (p = 0.50) or diet x dose interaction (p = 0.14) was observed.

3.1.4. Plasma AMH before FSH ovarian stimulation

Plasma AMH level, a predictor of the response to ovarian stimulation, was measured 9 days before FSH ovarian stimulation and was higher (diet effect, p = 0.044) in R ewes (113.8 ± 11.6 ng/mL) compared to WF ewes (88.6 ± 11.6 ng/mL), with no effect of the BPS dose (p = 0.71) or diet x BPS dose interaction (p = 0.44). Plasma AMH averaged 106.9 ± 16.7 pg/mL, 110.2 ± 16.7 pg/mL, 124.1 ± 16.7 pg/mL in R-0, R-4 and R-50 ewes respectively; 103.4 ± 16.7 pg/mL, 77.9 ± 16.7 pg/mL and 84.4 ± 16.7 pg/mL in WF-0, WF-4 and WF-50 ewes respectively; with no difference observed between the six groups of ewes (Tukey adjustment).

3.1.5. Plasma thyroid hormones

Plasma thyroid hormones were measured the day before the first OPU. For plasma free triiodothyronine (Fig. 5.A.), no effect of diet (p = 0.36) or BPS dose (p = 0.76) were observed, but a significant effect of the diet x BPS dose interaction was observed (p = 0.033). Plasma free triiodothyronine tended to be lower in R-4 ewes compared to WF-4 ewes (Tukey adjustment, p = 0.09). For plasma free thyroxine (Fig. 5.B.), no effect of diet (p = 0.13) or BPS dose (p = 0.60) were observed, but a tendency was nearly observed for the diet x BPS dose interaction (p = 0.107). Plasma total thyroxine (Fig. 5.C.) was lower in R ewes compared to WF ewes (diet effect, p < 0.0001), with no effect of BPS dose exposure (p = 0.28) or diet x BPS dose interaction (p = 0.69). R-0 ewes differed from WF-0, WF-4 and WF-50 ewes, R-4 ewes differed from WF-0, WF-4 and WF-50 ewes and R-50 ewes differed from WF-0, WF-4 and WF-50 ewes (Tukey adjustment, p < 0.05).

3.1.6. Ovarian follicular population

Two embryo production sessions by OPU-IVF were performed for all 60 ewes, that is to say 120 OPU-IVF sessions for a total of 1039 and 1079 follicles punctured in R and WF ewes, respectively. All punctured follicles were counted and their diameter was measured (Table 1). The total number of follicles averaged 17.7 ± 2.4 per OPU session in R ewes and 17.6 ± 2.4 in WF ewes (lsmeans ± SEM). No difference in the total number of follicles was found neither according to the diet (p = 0.94) nor according to the BPS dose (p = 0.95). The follicles were categorized into 3 categories according to their diameter: 2 mm, 3–5 mm and ≥ 6 mm. The absence of diet effect or BPS dose effect on the numbers and percentages (expressed as ratios in Table 1) of follicles was reported for all three categories. The effect of diet x dose interaction was significant for the percentage of 2 mm follicles (p = 0.03), and was observed as a tendency for the percentage of 3–5 mm follicles (p = 0.09) and the percentage of ≥ 6 mm follicles (p = 0.07).

3.1.7. Embryo production

Embryo production results are presented in Table 1. In this experimentation, the average numbers (lsmeans ± SEM) of cleaved embryos were 3.9 ± 0.6 per OPU session in R ewes and 3.0 ± 0.6 in WF ewes with corresponding cleaved embryo rates of 33.0 ± 5.2% and 25.4 ± 4.4%, respectively. The average numbers of blastocysts were 1.7 ± 0.3 per OPU session in R ewes and 1.6 ± 0.3 in WF ewes, and the corresponding blastocyst rates (normalised to the cleaved embryos) were 42.3 ± 7.1% and 48.4 ± 8.0%, respectively. No effect of the diet or of the BPS dose was observed on any of the parameters investigated in terms of number of embryos and developmental rates (Table 1). Nevertheless, a significant diet x BPS dose interaction was reported for both the number and the rate of cleaved embryos (p = 0.04 and p = 0.05, respectively), the number of > 4-cell embryos (p = 0.05), the number of blastocysts (p = 0.02) and the number of early blastocysts (p = 0.01).

Table 1
Effect of a 3-month BPS exposure on COC collection following ovum-pick up (OPU) and on *in vitro* embryo development (IVD) following *in vitro* maturation (IVM) and fertilisation (IVF), in restricted (R) or well-fed (WF) ewes. Results are presented as lsmeans ± SEM per experimental group.

	R (n = 30)			WF (n = 30)			Diet effect	Dose effect	Diet x Dose effect
	0 (n = 10)	4 (n = 10)	50 (n = 10)	0 (n = 10)	4 (n = 10)	50 (n = 10)	P-value	P-value	P-value
Nb punctured follicles	18.5 ± 2.7	16.4 ± 2.7	18.2 ± 2.7	17.2 ± 2.7	18.3 ± 2.7	17.3 ± 2.7	0.94	0.95	0.54
Nb 2 mm follicles	9.3 ± 1.7	7.3 ± 1.7	9.3 ± 1.7	8.1 ± 1.7	10.7 ± 1.7	7.1 ± 1.7	1.00	0.85	*0.10*
% 2 mm follicles/punctured follicles	0.48 ± 0.08	0.41 ± 0.08	0.51 ± 0.08	0.48 ± 0.08	0.59 ± 0.08	0.4 ± 0.08	0.62	0.64	**0.03**
Nb 3–5 mm follicles	8.4 ± 1.5	7.3 ± 1.5	7.2 ± 1.5	7.4 ± 1.5	6.8 ± 1.5	8.1 ± 1.5	0.75	0.54	0.51
% 3–5 mm follicles/punctured follicles	0.47 ± 0.05	0.45 ± 0.05	0.39 ± 0.05	0.42 ± 0.05	0.36 ± 0.05	0.47 ± 0.05	0.53	0.53	*0.09*
Nb ≥ 6 mm follicles	0.7 ± 0.6	1.8 ± 0.6	1.6 ± 0.6	1.6 ± 0.6	0.9 ± 0.6	2.1 ± 0.6	0.75	0.27	0.13
% ≥ 6 mm follicles/punctured follicles	0.05 ± 0.04	0.14 ± 0.04	0.1 ± 0.04	0.1 ± 0.04	0.05 ± 0.04	0.13 ± 0.04	0.86	0.40	*0.07*
Nb recovered COC	11.1 ± 1.8	10.5 ± 1.8	11.5 ± 1.8	10.4 ± 1.8	12.4 ± 1.8	10.5 ± 1.8	0.94	0.87	0.49
% recovered COC/punctured follicles	0.6 ± 0.05	0.66 ± 0.05	0.64 ± 0.05	0.6 ± 0.05	0.69 ± 0.05	0.62 ± 0.05	0.95	0.32	0.88
Nb oocytes in IVM	11.4 ± 1.8	10.3 ± 1.8	11.1 ± 1.8	10 ± 1.8	12.4 ± 1.8	10.1 ± 1.8	0.93	0.81	0.35
% oocytes in IVM/recovered COC	1 ± 0.02	0.98 ± 0.02	0.96 ± 0.02	0.96 ± 0.02	0.99 ± 0.02	0.97 ± 0.02	0.39	0.53	0.18
Nb cleaved embryos	4.7 ± 0.9	2.5 ± 0.9	4.5 ± 0.9	2.1 ± 0.9	4.3 ± 1	2.6 ± 0.9	0.22	0.99	**0.04**
% cleaved embryos/oocytes in IVM	0.39 ± 0.09	0.23 ± 0.07	0.39 ± 0.09	0.2 ± 0.06	0.35 ± 0.08	0.22 ± 0.06	0.19	0.98	**0.05**
Nb Embryos > 4-cells	2.9 ± 0.7	1.6 ± 0.7	3 ± 0.7	1.6 ± 0.7	3.2 ± 0.7	1.5 ± 0.7	0.51	0.96	**0.05**
% Embryos > 4-cells/cleaved embryos	0.61 ± 0.09	0.61 ± 0.1	0.65 ± 0.09	0.77 ± 0.09	0.76 ± 0.07	0.55 ± 0.11	0.27	0.42	0.19
Nb blastocysts	2.2 ± 0.6	0.9 ± 0.6	2.1 ± 0.6	1.2 ± 0.6	2.8 ± 0.6	1 ± 0.6	0.83	0.87	**0.02**
% blastocysts/cleaved embryos	0.51 ± 0.12	0.36 ± 0.12	0.41 ± 0.12	0.54 ± 0.14	0.69 ± 0.11	0.24 ± 0.11	0.57	0.18	0.12
Nb early blastocysts (non expanded)	1.7 ± 0.4	0.5 ± 0.4	1.2 ± 0.4	0.7 ± 0.4	2.1 ± 0.4	0.6 ± 0.4	0.95	0.59	**0.01**
% early blastocysts /cleaved embryos	0.37 ± 0.09	0.19 ± 0.08	0.23 ± 0.08	0.33 ± 0.11	0.47 ± 0.1	0.17 ± 0.07	0.48	0.21	0.12
Nb expanded blastocysts	0.5 ± 0.3	0.4 ± 0.3	0.9 ± 0.3	0.5 ± 0.3	0.7 ± 0.3	0.4 ± 0.3	0.71	0.79	0.42
% expanded blastocysts/cleaved embryos	0.1 ± 0.05	0.14 ± 0.07	0.17 ± 0.07	0.17 ± 0.09	0.15 ± 0.06	0.08 ± 0.05	0.93	0.90	0.49

Nb: number; COC: cumulus oocyte complex; bold text indicate significant difference p < = 0.05; italicised text indicate tendency 0.05 < p < = 0.10; linear mixed models or logistic regression mixed models were performed to analyze numbers and rates, respectively, multiple comparisons of lsmeans estimated by the models were performed using a Tukey adjustment for all parameters.

4. Discussion

To the best of our knowledge, the present work is the first to assess whether the metabolic status could affect ovarian sensitivity to the effects of endocrine disruptors. The results obtained in the ewe model suggested that a chronic diet exposure to BPS 4 or 50 μg/kg/day would not alter the oocyte quality. Nevertheless, the significant effect of diet x BPS dose interaction reported regarding embryo production parameters suggested that according to the metabolic status of the animal, the effect of BPS would not be the same. Moreover, the difference in metabolic status also affected the steroid secretion in the ewe, notably the plasma progesterone level, suggesting corpus lutea of lower quality in restricted ewes which could therefore related to higher embryo losses due to an implantation issue.

4.1. Diet effect

The diet plan used in this experiment was effective independently of BPS exposure. We indeed reported significant differences between R and WF ewes in terms of BCS and BW as expected. The significant diet effect observed for the metabolic parameters, plasma glucose being lower and plasma NEFA higher in the R ewes, were also relevant with the difference of BCS and BW. Indeed, R ewes were expected to consume glucose and mobilise their body lipid reserve more actively to reach their energy requirement, which would decrease plasma glucose and increase their plasma NEFA levels. The differences in metabolic status were similar to those reported in a previous study using the same diet plan (Menassol et al., 2012). No differences in terms of embryo production parameters were observed between R and WF ewes. Before FSH stimulation, there was a clear decrease in plasma progesterone levels in R ewes compared to WF ewes. This difference in progesterone was reported while ewes where synchronised and therefore during the luteal phase (12 days after the second cloprostenol injection). Different non-exclusive hypotheses could explain this result. First, the reduced plasma progesterone level reported in R ewes could suggest a lower quality of corpus luteum (CL, critical for the maintenance of reproductive cyclicity and pregnancy support) that would secrete less progesterone. A reduced plasma progesterone level was previously reported in underfed ewes compared to overfed ones (Kaminski et al., 2015). But overfeeding was also reported to reduce circulating progesterone (McEvoy et al., 1995). Such a reduction in the progesterone level could potentially affect fertilisation and the embryo implantation stage, therefore impairing the fertility of ewes (Boland et al., 2001). This decrease in progesterone may also suggests that the metabolic status affected the ovulation rate or the length of the luteal phase in ewes. Indeed, if less ovulation occurred in R ewes, a smaller number of corpus luteum would be generated which would lead to a potentially lower level of plasma progesterone. Such diet effect, with a lower corpus luteum number has already been reported in underfed non superovulated ewes compared to overfed ewes (Kraisoon et al., 2018). The plasma progesterone was measured at the end of the luteal phase so few days before the physiological decrease in progesterone level. A shortening of the luteal phase in R ewes would thus appear as a reduction in progesterone level at the moment of the blood collection. It would be interesting to perform progesterone measurements more frequently on 2–3 oestrous cycles with 3 measurements per week, and to monitor the number of ovulations using ultrasonography, in order to confirm whether this difference in progesterone level is observed during the entire luteal phase and whether the ovulation rate is affected by the metabolic status. It would also be interesting to assess the steroidome of ewes to compare R and WF ewes and to investigate whether the secretion of other steroids are also affected. All the differences reported with significant diet effects are therefore not related to the BPS exposure. Further studies would be required to elucidate how the metabolic status difference leads to the reduction in progesterone level and whether it affects fertility and/or prolificacy of ewes.

4.1.1. Bisphenol S exposure

The absence of an effect of chronic oral BPS exposure on the ovarian $>$ 2 mm follicular population following FSH stimulation and on the ewe oocyte quality and on the ovarian follicular population was reported in the present study. This result is different to that which was previously reported in mice, where BPS altered the cleavage rate and reduced the amount of ovarian follicles (Nevoral et al., 2018). These effects in mice were reported after BPS oral exposure of 10–100 μg/kg/day, which is comparable to the dose assessed in the present paper (4–50 μg/kg/day) but without FSH stimulation. Nevertheless, our study was performed in primiparous adult ewes and FSH stimulated for follicle growth. In addition, BPS was mixed with the diet while mice were around puberty and BPS was provided in drinking water (Nevoral et al., 2018). This discrepancy between results is probably not due to a difference in BPS clearance. Indeed, it was already reported that the BPS clearance is similar between rodents (rat) and pigs or sheep (Gayrard et al., 2019). As the actual plasma BPS and BPS-g levels are not always measured in reproductive studies relying on oral BPS exposure, it is possible that differences in BPS gastro-intestinal absorption or in the intensity of the first-pass effect might occur between species, therefore potentially leading to difference in the plasma level of unconjugated BPS and consequently to difference in ovary BPS exposure. We thus hypothesised that the BPS level assessed in the present study was low. It indeed reached up to 2.95 nM unconjugated BPS 1.5 h after oral exposure, which is the previously reported optimal assessment time (Gayrard et al., 2019). Nevertheless, the oral BPS exposure used in the present study is relevant to the TDI (50 μg/kg/day), and temporary TDI (4 μg/kg/day) defined for BPA in humans. The plasma BPS levels measured in the present study are also relevant compared to actual human exposure (Liao et al., 2012). Even if there is variability between animals, the plasma BPS and BPS-g levels are in accordance with the oral dose received (either 4 or 50 μg/kg/day). Our data therefore suggested that in adult females, chronic exposure lower than 3 nM of unconjugated BPS did not impair the follicular population after FSH stimulation and the oocyte quality in terms of early developmental competence (maturation, fertilisation and development up to the blastocyst stage). It would be interesting to assess the embryo cell number to compare the blastocyst quality of exposed or unexposed ewes. The absence of a chronic effect of BPS during folliculogenesis on oocyte quality is different to that which was reported after acute *in vitro* BPS exposure in COCs. Indeed, we previously reported that a 24 h BPS exposure at nanomolar concentration during *in vitro* oocyte maturation led to a decrease in blastocyst rate and therefore affected oocyte developmental competence in the ewe (Desmarchais et al., 2020). This *in vitro* study was the opposite of the present *in vivo* study. Indeed, in the *in vitro* study, the acute effect of BPS was only investigated during late maturation, while in the present study, the chronic effect of BPS was investigated in the entire folliculogenesis period, except for the last step of *in vitro* maturation. These data could suggest that the *in vitro* maturation stage is more sensitive to the effects of BPS, thus explaining why oocyte competence was affected. It could also suggest that BPS used during *in vitro* maturation was not conjugated, while *in vivo* bisphenol A, S or AF conjugation to inactive forms, that do no exert oestrogenic properties (Skledar et al., 2016), occurred rapidly, mainly in the liver. Such differences in BPS metabolism could explain the difference in the effects observed between *in vitro* and *in vivo* studies. Nevertheless, it is possible that, in the present study, the ovarian stimulation used, by strongly regulating steroidogenesis, might have masked potential BPS effects on the ovary. It is also possible that the absence of difference observed on the $\geq$ 2 mm antral follicles might not reflect the BPS effect on smaller follicle sizes. It would therefore be interesting to investigate pre-antral follicles. Lastly, it is important to note that the absence of an effect on oocyte quality does not mean a total absence of effect. Oocytes could indeed be affected by BPS exposure at an epigenetic level (Nevoral et al., 2018). Changes in methylation of DNA and histone H3 were indeed reported in mice after a long term exposure. Such epigenetic changes are

also observed in studies reporting BPA induced alteration of core histones H3 and H4 methylation in oocytes (Trapphoff et al., 2013). These epigenetic modifications won't necessarily lead to alterations in embryo developmental rates, which we assessed in the present study, but could lead to deleterious consequences later in life. It would therefore be interesting, in future studies, to investigate such modifications that could potentially affect late embryo, foetal or post-natal development. This is especially important because women can be exposed for years or decades to BPS. It would also be interesting to investigate the potential ovarian effects of the BPS on a more sensitive model. BPS exposure could be performed on pregnant ewes to assess its potential chronic effects on ovarian development of foetuses exposed *in utero* and on their reproductive performance once they would reach adulthood.

4.1.2. Interaction between BPS exposure and the metabolic status

Interestingly, despite the absence of a simple effect of the BPS exposure on oocyte quality reported in the present study, a significant effect of diet x BPS dose interaction was observed on several follicular population and embryo production parameters (ratio of 2-mm follicles, number of cleaved embryos, of >4-cell embryos, of blastocysts, of unexpanded blastocysts and rate of cleaved embryos), as well as on plasma T3 level. Regarding T3 level, an absence of the effect of overfeeding and underfeeding has already been reported in sheep (Grazul-Bilska et al., 2012), therefore suggesting that the effect observed in the present study is linked to the combination of both the metabolic status and the BPS exposure. The thyroid hormones exert mitogenic and pro survival effects on ovarian follicular cells (Canipari et al., 2019). Nevertheless, it is not possible to decipher whether the variation in T3 levels is among causes or consequences of the variation in embryo production, as ovarian stimulation can affect thyroid function (Mintziori et al., 2016). The fact that the BPS effect is opposite depending on the metabolic status could also contribute to explain why a simple BPS effect was not reported in the present study. A significant effect of diet x BPS dose interaction suggested that the effect of BPS varied according to the metabolic status of the animal. This possibility corresponds to our hypothesis that metabolic status could modify the sensitivity of ovarian cells to the effects of BPS. This potential change in sensitivity might rely on two separate explanations. First, the sensitivity of cells is affected by the level of circulating lipids that vary according to metabolic status and that can directly impair cell viability. Second, modulations of intracellular glucose or lipid metabolism can modify ovarian cell functioning. It was previously reported that exacerbated lipid metabolism impairs the oocyte quality (Leroy et al., 2014), and that in order to protect the oocyte, the cumulus cells internalise lipids but reduce their viability in this process (Aardema et al., 2017). On the other hand, it is also possible that BPS led to subtle glucose or lipid metabolism changes. Indeed, BPA and BPS exposure were shown to be related to obesity and type 2 diabetes (Rancière et al., 2019). Modifications in glucose and especially in insulin could affect ovarian cells and oocyte competence (for review, (Fontana and Torre, 2016)). Moreover, recent studies reported that BPA and BPS were able to impair the lipid metabolism in the rat or fish (Tonini et al., 2020), to induce lipid accumulation in mouse adipocytes (Ramskov Tetzlaff et al., 2020) and in HepG2 cells (Song et al., 2019), to enhance adipogenic signalling pathway in human mesenchymal stem cells (Salehpour et al., 2020), to regulate the PPARγ pathway and to worsen the metabolic outcomes of a high fat diet in mouse liver (Figueiredo et al., 2020). It has previously been reported that regulations in intracellular lipid metabolism are able to modulate ovarian cell functions and to influence oocyte competence (Dunning et al., 2014). In the present study, ewes exhibiting contrasted metabolic status would also likely exhibit differences in intra-follicular lipid metabolism, in terms of fatty acid level, lipogenesis or lipolysis state. Such pre-existing metabolic status differences, could contribute to explain why a BPS exposure might not lead to the same intracellular metabolism modifications and therefore might not lead to the same effect on the ovarian cell functions. Nevertheless, to properly assess such hypothesis, it would require an experimental design including more animals and exacerbating the difference in metabolic status. BCS would need to be more contrasted between groups, which is an aim that is not easy to reach because the diet has to remain compatible with animal welfare. It would also require a longer period of experimentation to reach contrasted BCS and BW. To check whether The BPS exposure dose used to assess this hypothesis of a difference of sensitivity between animals would also need to be higher or longer so that in extreme group a clear phenotype could be observed.

As discussed above, this experimentation presented several limitations. Even if the number of animals used in this experiment was quite high, an even higher number of animals would strengthen the study. Moreover, BPS was provided through the diet in order to be more comparable to human exposure. Nevertheless, it also led to individual differences in BPS exposure between ewes of the same treatment group, therefore potentially inducing variability in the parameters recorded. The sensitivity of the BPS assay is high but our low dose exposure conditions corresponded to undetectable unconjugated BPS (below the limit of quantification). Nevertheless, BPS-g levels were above the limit of quantification in all ewe groups exposed to BPS. Lastly, as mentioned above, more contrasted metabolic status between R and WF ewes, together with higher or longer exposure BPS doses would render it possible to properly assess the interaction between metabolic status and the sensitivity of ovarian cells to BPS exposure.

5. Conclusion

Our results suggested that a chronic diet exposure to BPS 4 or 50 μg/kg/day would not alter the oocyte quality. Nevertheless, the effect of diet x BPS dose interaction reported regarding the embryo production parameters and T3 thyroid hormone suggested that the metabolic status of the animal might modify the effect of BPS. Moreover, in restricted ewes, the plasma progesterone level was lower, suggesting corpus lutea of lower quality that could therefore be related to higher embryo losses due to implantation issues. Further studies are required to investigate the hypothesis on the metabolic status on more contrasted animals and with higher BPS exposure doses.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

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