Animal 19 (2025) 101392

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

The walnut tree as a source of progesterone for reproductive control in goats

P. Chemineau [a,*], A.L. Lainé [a], D. Gennetay [a], C. Porte [a], D. Chesneau [a], C. Laclie [a], G. Goudet [a], M. Meunier [a], M. Delmas [b], M.L. Greil [b], P. Liere [c], A. Pianos [c], A. Bernard [d], E. Dirlewanger [d], J.A. Delgadillo [e], M. Keller [a]

[a] Physiologie de la Reproduction et des Comportements, CNRS, INRAE, Université de Tours, 37380 Nouzilly, France
[b] INRAE, UEA, 33210 Toulenne, France
[c] U1195 INSERM Université Paris Saclay, Kremlin Bicêtre, France
[d] INRAE, Université de Bordeaux, UMR BFP, 33882 Villenave d'Ornon, France
[e] Centro de Investigación en Reproducción Caprina (CIRCA), Universidad Autónoma Agraria Antonio Narro, 27054 Torreón, Coahuila, Mexico

ARTICLE INFO

Article history:
Received 20 August 2024
Revised 28 November 2024
Accepted 29 November 2024
Available online 4 December 2024

Keywords:
Male effect
Oestrus
Ovulation
Plants
Small ruminants

ABSTRACT

Intravaginal sponges impregnated with the progesterone (**P4**) analogue fluorogestone acetate (**FGA**) induce synchronous oestrous behaviour and normal ovulatory cycle in goats. To explore alternatives using natural P4 from plants, we developed a method of ethanolic extraction and a specific enzyme immunoassay (**EIA**) to measure P4 in the different parts of the walnut tree *Juglans regia*. We found a very high concentration of P4, specifically in the leaves of the three most common French varieties (~100 mg/kg of DM) but not in flowers, fruits, septa, husk, oil or cake. High concentrations of P4—and to a lesser extent its reduction metabolites and phytosterols—were also measured by Gas Chromatography-Mass Spectrometry/Mass Spectrometry in leaf extracts. P4 concentrations were five times higher in October than in June. P4 was detected in 182 varieties of *Juglans regia* ranging from 35 to 287 mg of P4 per kg of leaf DM. We collected large quantities of leaves over 6 years, which were used to manufacture feed pellets containing 32% of dry leaf for distribution to female goats. To determine their dietary acceptance and their efficacy in terms of P4 blood plasma concentration, three trials in ovariectomised goats and four trials in ovary-intact goats were performed (N = 83). The distribution of 600 g of pellets per day per ovary-intact goat over 3 days, 6 and 4 days before the introduction of males in April allowed us to achieve our objective of a significant increase of P4 plasma concentration to ~1.5 ng/mL measured by EIA from 24 to 72 h after the first distribution in the walnut pellet group (n = 13). The two control groups of goats (FGA, n = 12 and control, n = 10) showed no increase in plasma P4. However, despite this high P4 plasma concentration, goats of the walnut group had the same percentages of goats in oestrus at the first ovulation and of goats experiencing short ovulatory cycles after introduction of males (54 and 77%, respectively) as the group of control goats (80 and 90%), whereas the FGA goats showed very different percentages (100 and 0%, $P < 0.01$). It was concluded that whereas walnut leaves contain a high concentration of P4—and its reduction metabolites and phytosterols—the pellet feeding mode does not allow for restoration of oestrus behaviour and duration of the induced cycle consistently achieved with FGA-impregnated intravaginal sponges.

Implications

The objective is the need to replace fluorogestone acetate treatments by natural progesterone associated with the male effect in goats. We showed that walnut leaves of *Juglans regia* contain very high concentrations of progesterone (up to 287 mg/kg of dry leaves). The distribution of pellets containing walnut leaves to goats allowed them to reach physiological levels of progesterone in their blood (≥ 1 ng/mL of plasma over 48 h). However, this was insufficient to induce oestrus at the first ovulation and to suppress short cycles after the introduction of males, as is consistently observed in goats receiving fluorogestone acetate–impregnated intravaginal sponges.

Introduction

In small ruminants, the synchronous induction of ovulation and oestrus is based on a progestogen-only phase comprising a vaginal sponge impregnated with a progesterone (**P4**) analogue, generally

* Corresponding author.
E-mail address: Philippe.Chemineau@inrae.fr (P. Chemineau).

https://doi.org/10.1016/j.animal.2024.101392
1751-7311/

fluorogestone acetate (**FGA**) or medroxyprogesterone acetate, left in place for about 5 (Menchaca et al., 2007) to 10 days (Chemineau et al., 1991). When the sponge is removed, an injection of equine chorionic gonadotropin (**eCG**), also called pregnant mare serum gonadotropin, stimulates the ovary and secretion of oestradiol. The P4 analogue–eCG sequence thus leads to the expression of oestrous behaviour and of normal ovulation within 48 h of injection in all females. High fertility rates ($\geq$ 65% of parturient females after a single artificial insemination) are achieved in sheep and goats. Widely used on millions of animals worldwide each year for more than 40 years, this treatment has been proven to achieve spring fertilisation in seasonal sheep and goat populations, thereby providing technical and economic advantages. The genetic improvement of flocks, particularly dairy flocks, has been largely based on this technique, which makes it possible to fertilise many animals from the same flock on the same day with the semen of progeny testing and/or genetically improved males (Buisson et al., 2018).

However, for several reasons, this treatment is currently being questioned. The use of synthetic analogues of P4 that do not exist in nature leads to a risk of environmental pollution, the animal welfare conditions under which eCG is produced have been criticised and the marketing authorisations for these products are generally historic, and it is not certain that their renewal would be as easy now as it was previously. In addition, the insertion of vaginal sponge may provoke changes in bacterial vaginal populations (Suarez et al., 2006; Gatti et al., 2011) which may lead to affect negatively female sexual attractiveness (Ungerfeld and Silva, 2005), sperm quality (Manes et al., 2016) and, at the end, conception rate (Manes et al., 2014). Finally, these treatments are prohibited in organic farms, preventing them from fully benefiting from out-of-season lambing and kidding, and from the progress made in flock genetic improvement.

In the FGA sponge–eCG sequence, the replacement of eCG by the introduction of sexually active males (the 'male' effect) previously subjected to photoperiod treatment during winter allows goat fertility rates equivalent to those of FGA–eCG treatment (Pellicer-Rubio et al., 2008 and 2016). However, to our knowledge, no alternative currently exists to replace the first, progestogen-only, part of the treatment with products not derived from synthetic chemistry.

Using P4 extracted from plants is an attractive alternative and we are interested in three plants said to contain concentrations that may be as close to the minimum effective P4 dose in sheep and goats (~25 mg by intramuscular injection) (Andrade-Esparza et al., 2019; Oldham et al., 1985; Pearce et al., 1985). The false rubber tree *Holarrhena floribunda* (Bennett and Heftmann, 1965; Leboeuf et al., 1969), the Malayan mistletoe ('benalu duku') *Dendrophthoe pentandra* (Lazuardi and Hermanto, 2016; Lazuardi et al., 2019) and the walnut tree *Juglans regia* (Pauli et al., 2010) all seemed promising candidates with claimed P4 concentrations of between 1 (Pauli et al., 2010) and 57 g/kg dry leaves (Lazuardi and Hermanto, 2016; Lazuardi et al., 2019). However, in preliminary work using an enzyme immunoassay (**EIA**), we were unable to replicate the literature values of the first two species (*Holarrhena floribunda*: Rubio-Pellicer, Maria-Teresa, personal communication, and *Dendrophthoe pentandra*: Supplementary Material S1). In contrast, our preliminary work with walnut tree leaves demonstrated concentrations close to or even higher than those reported by Pauli et al. (2010). Importantly, the walnut tree is indigenous to temperate zones. It is the dominant nut tree grown in France in terms of orchard area, and we had access to a collection of several hundred varieties of the *Juglans* genus held by the Institut national de recherche pour l'agriculture, l'alimentation et l'environnement (INRAE) (Bernard et al., 2018 and 2021).

The results presented below were therefore obtained from walnut trees. Our working hypothesis was that it might be possible to use walnut products to increase plasma P4 in goats to the physiological level necessary to induce oestrus behaviour and suppress short cycles after exposure to males. After developing a simple and effective extraction method, we determined the concentration of P4 in different parts of the walnut tree by EIA and also determined its direct (pregnenolone) and potential precursors (phytosterols), and its metabolites in walnut leaves by Gas Chromatography-Mass Spectrometry/Mass Spectrometry (**GC–MS/MS**). Second, we explored the effects of different environmental and genetic factors that affect P4 concentration in walnut leaves. Third, we explored the possibility of feeding walnut leaves to goats to achieve a blood plasma concentration close to the physiological concentrations observed after ovulation. Finally, we tested the clinical efficacy of the best mode of distribution, compared with the conventional treatment of FGA-impregnated sponges, on oestrous behaviour and on the duration of the ovulatory cycle induced by the male effect in spring.

Material and methods

A schematic representation of all experiments presented in this article is given in Fig. 1.

Plant material and genetic walnut resources

Plant sampling was made in the Fruit Experimental Unit of the INRAE Bordeaux research centre at Toulenne, located 50 km southwest of Bordeaux, France. All the chosen varieties were in this unit. They were planted between 1988 and 2000 from sources in 23 countries and are part of the Centre de Ressources Biologiques *Prunus-Juglans*, INRAE, member of Biological Resource Centres for plants-Ressources Agronomiques pour la Recherche, INRAE, 2022 (https://doi.org/10.17180/WN42-3J20; https://www.agrobrc-rare.org/). In the present study, we explored different walnut species present in the above collection: *Juglans regia* L., mostly used in France and abroad for its fruit production; *J. nigra* L., which is generally used for producing rootstocks and wood; and *J. major* (Torr.) A. Heller and *J. mollis* Engelm., which both originate from North America (Bernard et al., 2018).

Each walnut leaf sample comprised about 10 leaflets generally taken from adult trees managed for fruit or from pollards, sampled either at ground level or from a platform 3–5 m above ground. Leaves were air-dried at < 30 °C after collection using either a laboratory oven or a semi-industrial dryer used for walnut fruit. Drying generally took <48 h. Dry leaves were then stored in darkness at room temperature until needed. When not dried, fresh leaves were frozen and stored at − 20 °C within 24 h of collection.

Table 1 describes all walnut samples collected for the various experiments. In Year 1, walnut leaves, flowers, fruits, septa and husk were collected in May (flowers) and July (other tissues) from the three most common *Juglans regia* varieties in France, Fernor, Franquette and Lara. Samples were immediately immersed in 97% (v/v) ethanol to prevent degradation during transport and storage. Walnut leaves were collected in October to compare adult trees and pollards. Walnut oil, cake (remaining after oil extraction) and leaf powder originated from commercial sources. In Year 2, we aimed to determine the best month of leaf collection for P4 content. We collected leaves from five varieties (H92-28 Hybride INRA, RA11-95 Noyer pleureur, H101-2 Hybride INRA, H96-26 Hybride INRA, and RA-311 Franquette) monthly from June to October inclusive. Because we found that concentrations were five times higher in October than in June, all subsequent leaf collections were carried out in good weather conditions in the 1st week of October. In Year

Figure 1

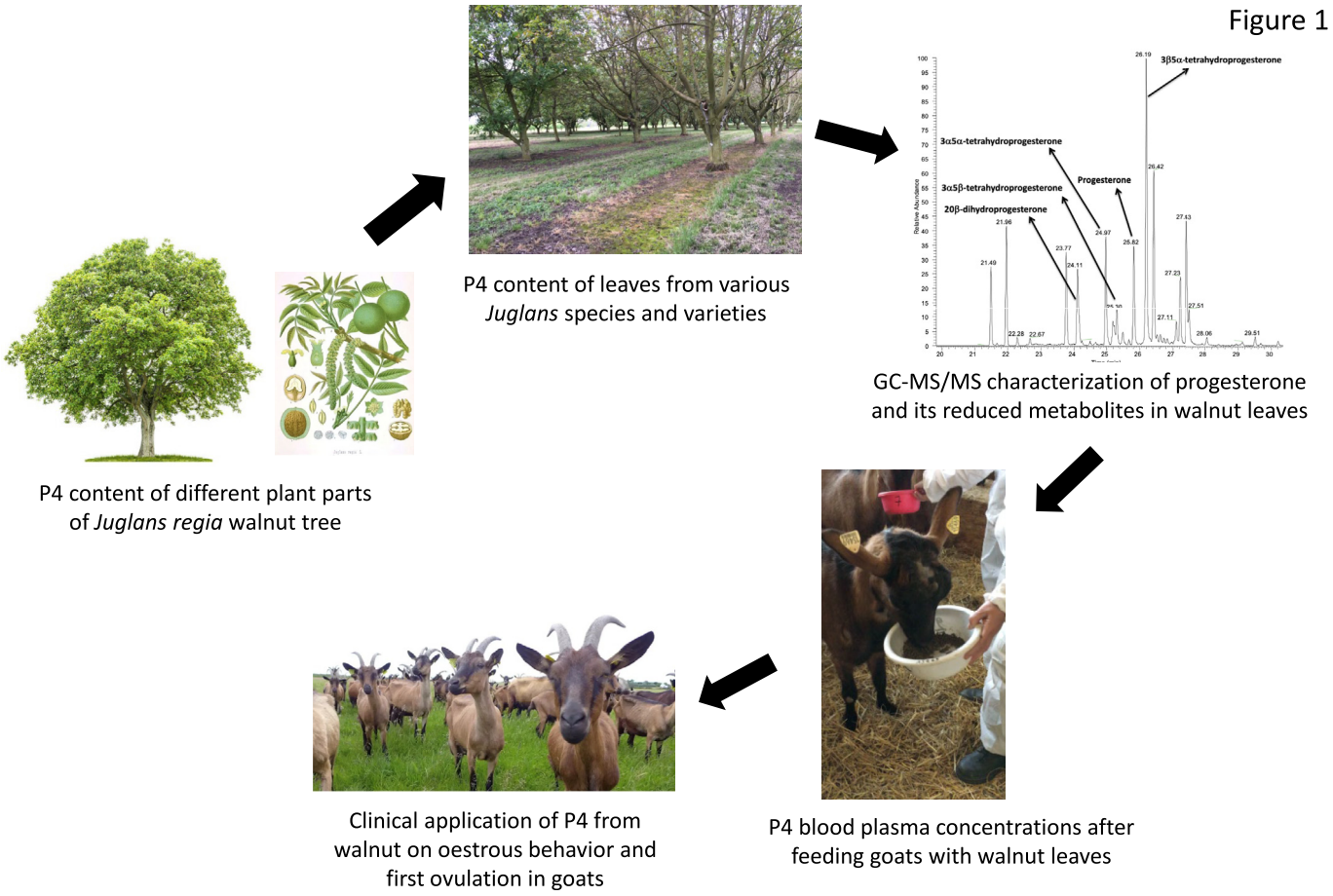

Fig. 1. Schematic representation of the successive experiments done to determine the effects of walnut tree as a source of progesterone for reproductive control in goats. P4: progesterone. GC–MS/MS: Gas Chromatography then tandem Mass Spectrometry.

Table 1
Overview of walnut samples taken for determination of their progesterone (P4) and P4 precursors or metabolite content used as a source of progesterone for reproductive control in goats.

Year	Objective(s)	Month(s) of collection	Assay methodology	Walnut variety	Samples
1	P4 content of different parts of the plant	(1) May, July, October (2) October (3) Unknown	EIA	(1) Fernor, Franquette, Lara (2) Fernor, Franquette, Lara (3) Unknown	(1) Leaves, flowers, fruits, septa, husk (2) Leaves from adult trees vs pollards (3) Oil, powder, cake
2	P4 seasonal variations	June–October	EIA	H92-28 Hybride INRA RA11-95 Noyer pleureur H101-2 Hybride INRA H96-26 Hybride INRA RA-311 Franquette	One sample of leaves per month
3	P4, P4 precursors, and metabolites	October	GC–MS/MS	Fernor, Franquette, Lara	Leaves
4	P4 phenotypic variability	October	EIA	189 varieties (182 from *Juglans regia*)	Leaves
5–7	Manufacture of pellets containing walnut leaves	October	EIA	Fernor, Franquette, Lara	Leaves from 21 pollard trees

P4 = progesterone; EIA = enzyme immunoassay; GC–MS/MS = gas chromatography coupled to tandem mass spectrometry. Samples (3) in Year 1 came from commercial sources.

3, leaves of Fernor, Franquette and Lara varieties were collected to perform the GC–MS/MS analyses. In Year 4, to determine the phenotypic variability of P4 concentration in leaves, we sampled a subset of walnut species and varieties over 2 days in October by collecting leaves from 189 varieties where 182 were from *Juglans regia* under the above conditions. Large quantities of walnut leaves of the Fernor, Franquette and Lara varieties were then collected in October for six consecutive years from the same 21 young trees managed as annually cut pollards to prepare different batches of leaf concentrate to be fed to goats.

Extraction and progesterone enzyme immunoassays

Various techniques of P4 extraction were tested with the dual objective of (1) accurately measuring the P4 content for research purposes and (2) using practical and non-harmful solvent(s) yield-

ing extracts that could be safely used in farms and/or in artisanal or industrial conditions. Different solvents (methanol, ethanol, and a mixture of cyclohexane and ethyl acetate) were tested on an analytical scale with frozen leaves. We settled on ethanol to extract dry leaves, where we fixed the ratio of dried leaf weight to ethanol volume and the duration of the extraction: gentle grinding of the dried leaves; weighing a 25 mg sample; extracting with 2 mL of ethanol; vortexing for 10 min; agitating at room temperature overnight; centrifuging for 10 min at 2 873 × g; evaporating under an air stream for 2 h; redissolving with Tris-BSA buffer (2 mL/tube) and storing at 4 °C until required for EIA. Under these conditions, the maximum extraction yield was 90%, calculated from two successive overnight extractions of the same samples. The final values were corrected for this bias.

Concentrations of progesterone were measured by EIA for blood plasma as described by Canépa et al. (2008) and using 25 µL of the final Tris-BSA solution. The method included reference samples of known values at regular intervals during the assay, which allowed us to estimate the CV and were used as quality controls. The EIA method was a competitive EIA in a 96-well plate. The plates were coated with goat anti-mouse IgG overnight at 4 °C. After a blocking step with Tris-BSA buffer, the plates were incubated overnight at 4 °C with progesterone-specific antibody and 25 µL of leaf extract in buffer. After incubation, a solution of progesterone alkaline phosphatase conjugate was added to each well and incubated in the dark for 1 h. Plates were washed with Tris-Tween 20 and then incubated with the visualisation reagent disodium p-nitrophenyl phosphate for 2 h at 37 °C. Absorbances were recorded at 405 nm with a plate reader (TECAN, Switzerland). The sensitivity was 0.25 mg/kg, and the interassay CV was < 5.8%. The specificity of this P4 antibody was tested against 27 P4-related molecules. Three compounds (5α-dihydroprogesterone, isopregnanonole, and 5β-dihydroprogesterone) cross-reacted at > 2% with our antibody (22, 15 and 14%, respectively; see Supplementary Material S2).

Analysis of steroids in walnut leaves by gas chromatography-mass spectrometry/mass spectrometry

The analytical protocol for steroid analysis in walnut leaves by GC–MS/MS—previously described for analyses of saliva and plasma from boar and mare (Goudet et al., 2019, 2021 and 2023)—was used here with minor modifications. Steroids were extracted from *Juglans regia* Fernor, Franquette and Lara varieties sampled in duplicate (Table 1), using 5 mg of dried ground leaves extracted with 1 mL of methanol. Internal standards (Téteau et al., 2022) were added to the walnut leaves for quantification of the endogenous steroids. The parameters of purification, fractionation, derivatisation, and analytical steps of the GC–MS/MS protocol were as described previously (Goudet et al., 2019, 2021 and 2023).

The analytical protocol was validated for steroids in terms of limits of detection, linearity, accuracy, and intra- and interassay precisions for all the targeted steroids using a pool of 1 mL of male mouse plasma (Téteau et al., 2022). The intra- and interassay CVs were 7.5 and 10.5%, respectively. The overall accuracy of the assay was 95–106%.

Manufacturing concentrate feed containing walnut leaves

Concentrate feed for ingestion by goats was manufactured in the INRAE Experimental Pig Unit in Saint-Gilles, France using an experimental mill. The final concentrate feed pellets contained 32% dried-then-ground walnut leaves, 65% barley, and 3% molasses. We verified that the barley and molasses did not contain P4. The pellet manufacturing process was carried out with the press temperature < 40 °C to avoid P4 degradation.

Progesterone blood plasma concentrations after walnut feeding, clinical trial to monitor the effects on oestrous behaviour and quality of ovulations after the male effect

Seven walnut-pellet feeding trials were conducted over 6 years. Three trials used ovariectomised females and four trials used females with intact ovaries; the ovariectomised goats were a control that eliminated any ovarian origin of P4. In total, we distributed the pellets to 83 goats and recorded any abnormality in feeding behaviour or any other unexpected behaviour or health problem. Control groups of goats received commercial pellets without walnut leaves.

Table 2 summarises the experiments. In all experiments with ovary-intact goats, we monitored P4 blood plasma concentrations before the start of the experiment, and ignored cyclic and pseudo-pregnant goats so as not to confound endogenous and exogenous P4 determinations. In all experiments, we followed the blood plasma concentration of P4 before, during and after pellet distributions. Blood sampling frequency was adapted to the observation we needed: twice-weekly collections to determine if females were cycling and daily collections to follow the P4 secretion of the *corpus luteum* after ovulation and/or P4 from the pellets passed into the blood. All blood samples were collected by jugular venepuncture in 4-mL tubes containing 83 international units of heparin. Plasma was obtained after centrifugation at 2 500 × g for 20 min and stored at −20 °C until hormone determinations.

Experiment 1 was performed to ascertain if feeding ovary-intact anoestrus goats with 250 g of walnut pellets every morning for 5 consecutive days would increase P4 blood plasma concentration. All goats of the five groups were blood sampled 1 h before the first pellet distribution or injections/ingestions, then subsequently at 1, 2, 4, 8 and 12 h on Day 0, and then every morning and afternoon at 24, 31, 48, 55, 72, 79, 96 and 103 h (end of Day 5). Experiment 2 was performed in ovariectomised goats to eliminate the ovarian origin of P4 detected in the blood. The three groups in that experiment were blood sampled on the same schedule as in Experiment 1. In Experiment 3, also using ovariectomised goats, our objective was to simplify the treatment. We wanted to know if halving the duration of distribution while simultaneously doubling the number of daily walnut pellets distributed was feasible in terms of behavioural acceptance of feed and if there was an effect in terms of blood plasma P4 concentration. Thus, we distributed 250 g of walnut pellets every morning and evening for 2.5 consecutive days and monitored the P4 blood plasma concentration. All goats of the three groups were blood sampled on the same schedule as in Experiment 1. In Experiment 4, we investigated (1) if goats were able to eat the same total quantity of walnut pellets as in Experiment 3 (i.e., 1 250 g) during a single day without behavioural or health disorders and (2) if there was an effect in terms of blood plasma P4 concentration. We distributed 1 250 g of walnut pellets to ovariectomised goats in a single day and monitored their pellet intake. We followed the P4 blood plasma concentration, in comparison with goats receiving 0 or 100 mg of P4 by oral supplementation in a single application at 0900 h on Day 0. All goats of the three groups were blood sampled on the standard schedule. The four experiments used a Latin square design schedule so that all goats received all treatments.

The three following experiments (Experiments 5–7) were carried out with (ovary-intact) anoestrus goats, where the objective was to define a pellet distribution protocol that was both practicable and effective. Thus, we varied the duration of distribution (1, 3 and 3 days) and the interval between the last distribution and the introduction of males (3, 3, and 4 days beforehand) to ensure that the blood concentration of P4 was < 0.5 ng/mL at the introduction of males. In these three experiments, we monitored the effects of walnut pellet distribution on (1) blood plasma P4 and (2) oestrous

Table 2
Synthetic view of goat experiments raised to measure progesterone (P4) in the blood plasma after ingestion of pellets containing walnut leaves or synthetic P4 distributed per oral supplementation (os) or by intramuscular injection (im). Morning (m) = 0900 h; m + afternoon (a) = 0900 and 1900 h.

Experiment	Objective(s)	Reproductive status	Groups	Number of goats	Experimental design
1	Effect of feeding goats with walnut pellets for 5 days on plasma P4	Ovary-intact anoestrus goats	(1) 0 mg P4 (2) 25 mg P4 per os (3) 50 mg P4 per os (4) 25 mg P4 im (5) 250 g walnut pellets, m, for 5 days	16	Latin square (i.e. all goats received all treatments)
2	To discard any ovarian origin of P4	Ovariectomised goats	(1) 0 mg P4 (2) 25 mg P4 per os (3) 250 g walnut pellets, m for 5 days	11	Latin square
3	To shorten the duration of the treatment: 2.5 vs 5 days	Ovariectomised goats	(1) 0 mg P4 (2) 100 mg P4 per os (3) 250 g walnut pellets, m + a for 2.5 days	8	Latin square
4	To shorten the duration of the treatment: 1 vs 2.5 days	Ovariectomised goats	(1) 0 mg P4 (2) 100 mg P4 per os (3) 1 250 g walnut pellets for 1 day	12	Latin square
5	Is 1 day of walnut pellet distribution sufficient? Is walnut pellet distribution able to induce oestrus at first ovulation and suppress short cycles?	Ovary-intact anoestrus goats	(1) 0 mg P4 (2) FGA sponge for 11 days (3) 600 g walnut pellets for 1 dayInterval end walnut pellets – male = d-3	10 11 11	Independent groups
6	Interval end of distribution; introduction of males after 3 days Is walnut pellet distribution able to induce oestrus at first ovulation and suppress short cycles?	Ovary-intact anoestrus goats	(1) 0 mg P4 (2) FGA sponge for 11 days (3) 600 g walnut pellets for 3 daysInterval end walnut pellets – male = d-5 to d 3	11 14 12	Independent groups
7	Interval end of distribution; introduction of males after 4 days Is walnut pellet distribution able to induce oestrus at first ovulation and suppress short cycles?	Ovary-intact anoestrus goats	(1) 0 mg P4 (2) FGA sponge for 11 days (3) 600 g walnut pellets for 3 daysInterval end walnut pellets – male = d 6 to d 4	12 12 14	Independent groups

P4 = progesterone; FGA = fluorogestone acetate.

and ovulatory responses of ovary-intact out-of-season anoestrus goats to the introduction of sexually active males. For these three experiments, three independent but similar groups of goats were assembled based on age and BW. The control group (**C**) remained untreated and constituted the negative control. The FGA groups (FGA) received an intravaginal sponge impregnated with 45 mg of FGA (CEVA, Libourne, France) for 11 days, which was removed on the morning of the introduction of males among the groups. It constituted the positive control group. The walnut groups were fed 600 g of pellets per animal containing dry walnut leaves as previously described, over 1 day or 3 and 3 days before the introduction of males.

Unfortunately, and for unknown reasons, because males were not as sexually active as expected in the two first experiments, the females did not ovulate. So, while the data from the three experiments for P4 blood plasma concentrations after walnut pellet feeding were useful, only the oestrous and ovulatory response data from Experiment 7 were of value. Consequently, the results of Experiments 5 and 6 are shown in Supplementary Fig. S1. Sexually active males were introduced 96 h after the last walnut pellet distribution, based on the previous pharmacokinetic experiments. Control and FGA females received commercial concentrate.

Six males, previously treated with exposure to extra light followed by three melatonin implants (Melovine; CEVA) (Chemineau et al., 1992), were introduced to the females of the three groups (D0, 25 April of last year) in the ratio of two males to 15 females. The males were changed from one group to another every morning after oestrus detection. Males were permanently with females until the experiment ended. To guarantee the response to males in Experiment 7, all females of the three groups received an intramuscular injection of 500 international units of eCG (CEVA) immediately after sponge removal in the FGA group on Day 0. Oestrus detection was carried out every morning by recording marks left by male harnesses on the backs of female goats and by direct observation. Blood samples were taken daily from Day −7 to Day 17 for P4 assay to determine (1) blood concentration after pellet feeding, (2) if goats had ovulated and (3) the presence of short or normal ovulatory cycles.

Statistical analyses

Probability and chi-square tests were used to test for differences between proportions, and *t*-tests for differences between means. Samples were considered independent variables (Dagnelie, 1970; Snedecor and Cochran, 1971). Data are expressed as the mean ± SEM, and differences were considered significant at the level of $P \leq 0.05$. Grubbs' test was used to exclude outliers.

Results

Progesterone content of different plant parts of Juglans regia walnut tree and the effect of tree management

P4 was detected at very high concentrations (90–117.8 mg/kg of DM) (Table 3) in walnut leaves from Fernor, Franquette and Lara *Juglans regia* varieties. P4 concentration was below the limit of detection (0.25 mg/kg) in flowers, fruits, septa, powder, oil and cake, whereas the concentration in husk was just above this limit (Table 3). P4 content in walnut leaves did not differ significantly between trees cultivated as pollards or as adult trees (Table 3).

Progesterone content of various Juglans species and varieties

Very high levels of P4 in walnut leaves were specific to *Juglans regia* The other species tested, *J. nigra* (n = 2), *J. mollis* (n = 1) and *J. major* (n = 4), contained P4 of only 0.25–1.1 mg/kg of DM in leaves.

Table 3
Progesterone content determined by enzyme immunoassay in different parts of or products of *Juglans regia* walnut trees and the effect of tree management used as a source of progesterone for reproductive control in goats.

Plant parts (details of collection)	Number of samples	P4 concentration Mean ± SEM (mg/kg DM or mg/mL oil)
Flowers (May)	1	< 0.25
Fruits (June)	15	< 0.25
Septa (October)	2	< 0.25
Powder from leaves (unknown)	2	< 0.25
Cake (unknown)	4	< 0.25
Husk (October)	3	0.35 ± 0.12
Leaves from pollards (October)[a]	9	90.8[b] ± 13.4
Leaves from adult trees (October)[a]	9	117.8[b] ± 16.0
Oil (unknown)	2	< 0.6

P4 = progesterone.
[a] Leaves were collected over 3 years from Fernor, Franquette and Lara varieties.
[b] The P4 concentration was not significantly different between pollards and adult trees ($P > 0.05$).

In contrast, P4 concentrations of the 182 varieties of *Juglans regia* were all above the upper limit of quantification of the assay and were characterised by huge variability within this species. The P4 content ranged from 35 to 287 mg/kg of DM of leaves in the 182 walnut varieties in the INRAE Toulenne collection (Fig. 2).

Characterisation of progesterone and associated compounds by gas chromatography-mass spectrometry/mass spectrometry

GC–MS/MS analysis confirmed that walnut leaves contained very high concentrations of P4 (14.9 ± 2.4 mg/kg of DM) in the samples from Fernor, Franquette and Lara varieties of walnut leaves of *Juglans regia*. The analysis also showed that many other precursors (pregnenolone) and metabolites of P4, such as 5α-, 5β-, 20α- and 20β-reduced metabolites of P4, were present at high concentrations, ranging from 0.3 to 6 mg/kg of DM in walnut leaves (Table 4). Many other progestin compounds were also present in low concentrations. Regarding phytosterols, we detected very high concentrations of β-sitosterol and high concentrations of three other compounds: campesterol, stigmasterol and cholesterol (Table 4).

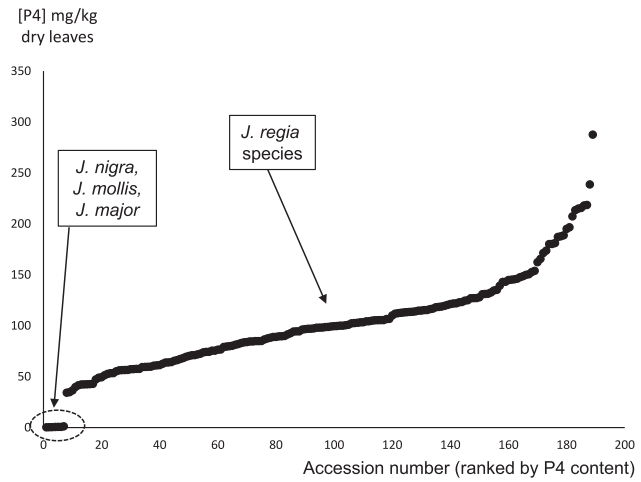

Fig. 2. Progesterone (P4) concentrations determined by enzyme immunoassay EIA (mean ± SEM in mg/kg DM) of walnut leaves in 182 *Juglans regia* varieties and three other *Juglans* species used as a source of progesterone for reproductive control in goats.

Progesterone blood plasma concentrations after feeding with walnut leaves

Except in only one experiment reported below, all females ate the walnut pellets that were offered. No eating or behavioural or health disorder was observed in any experiment. Distribution of walnut pellets every morning for 5 days in ovary-intact anoestrus goats (Experiment 1) induced a rapid increase in P4 blood plasma concentration to > 0.5 ng/mL as soon as 8 h after the first distribution of pellets on Day 0. The concentration reached 1.79 ng/mL 31 h after the first distribution, which was maintained between 1.25 and 1.79 ng/mL up to 103 h after the first distribution (Fig. 3a). The kinetics of the plasma concentrations in goats in the walnut group were very different from those of goats in the control group, which remained <0.30 ng/mL during the whole experiment (Fig. 3b). Goats receiving 25 or 50 mg of P4 as an oral supplement *Juglans regia* also showed a very rapid increase of P4 blood plasma concentrations to > 0.5 ng/mL as soon as 1 h after supplementation and remained > 0.5 ng/mL for up to 72 h (Fig. 3c and d). Goats receiving intramuscular injection of 25 mg of P4 showed a huge increase in P4 plasma concentration up to 12 ng/mL as soon as 1 h postinjection, which remained >0.5 ng/mL up to 55 h postinjection (Fig. 3e).

Distribution of walnut pellets every morning for 5 days in ovariectomised goats (Experiment 2) induced a rapid increase in P4 blood plasma concentration to > 0.5 ng/mL as soon as 8 h after the first distribution of pellets on Day 0, and reached 1.04 ng/mL 31 h after the first distribution; this high concentration was maintained between 0.66 and 1.00 ng/mL up to 103 h after the first distribution (Fig. 4a). A clear cyclicity appeared in mean P4 concentration (Fig. 4a) and in the four individual examples (Fig. 4d). The kinetics of plasma concentrations in goats in the walnut group were different from those of the control group goats, remaining < 0.14 ng/mL during the entire experiment (Fig. 4b). Goats receiving 25 mg of P4 orally also showed a rapid increase of P4 blood plasma concentrations to > 0.4 ng/mL as soon as 4 h after oral administration and remained around 0.12 ng/mL up to 31 h after the first distribution (Fig. 4c).

Table 4
Concentration of progesterone, its main direct precursor (pregnenolone), its metabolites and four phytosterols in walnut leaves by GC–MS/MS. Leaves were collected in October from eight (progestins) or six (phytosterols) trees of Fernor, Franquette and Lara varieties of *Juglans regia*. Concentrations are expressed as mg/kg of DM of walnut leaves used as a source of progesterone for reproductive control in goats. Values are mean ± SEM (n = 8 or 6). Grubbs' test was used to exclude outliers.

Compounds	Concentration (mg/kg)
Progestins	
Progesterone	14.9 ± 2.4
5α-Dihydroprogesterone	11.5 ± 1.8
5α,20β-Tetrahydroprogesterone	8.1 ± 4.2
5β-Dihydroprogesterone	5.7 ± 0.7
20α-Dihydropregnenolone	4.5 ± 0.8
3α,5β-Tetrahydroprogesterone	1.7 ± 0.5
20β-Dihydroprogesterone	1.2 ± 0.3
20α-Dihydroprogesterone	0.8 ± 0.2
Pregnenolone	0.6 ± 0.2
3α,5α-Tetrahydroprogesterone	0.5 ± 0.2
Androstenedione	0.4 ± 0.1
3α,5α-Tetrahydroprogesterone	0.3 ± 0.1
5α,20α-Tetrahydroprogesterone	0.3 ± 0.2
Phytosterols	
β-Sitosterol	896.1 ± 69.2
Campesterol	8.4 ± 1.4
Stigmasterol	5.5 ± 1.0
Cholesterol	2.1 ± 0.4

GC–MS/MS = gas chromatography coupled to tandem mass spectrometry.

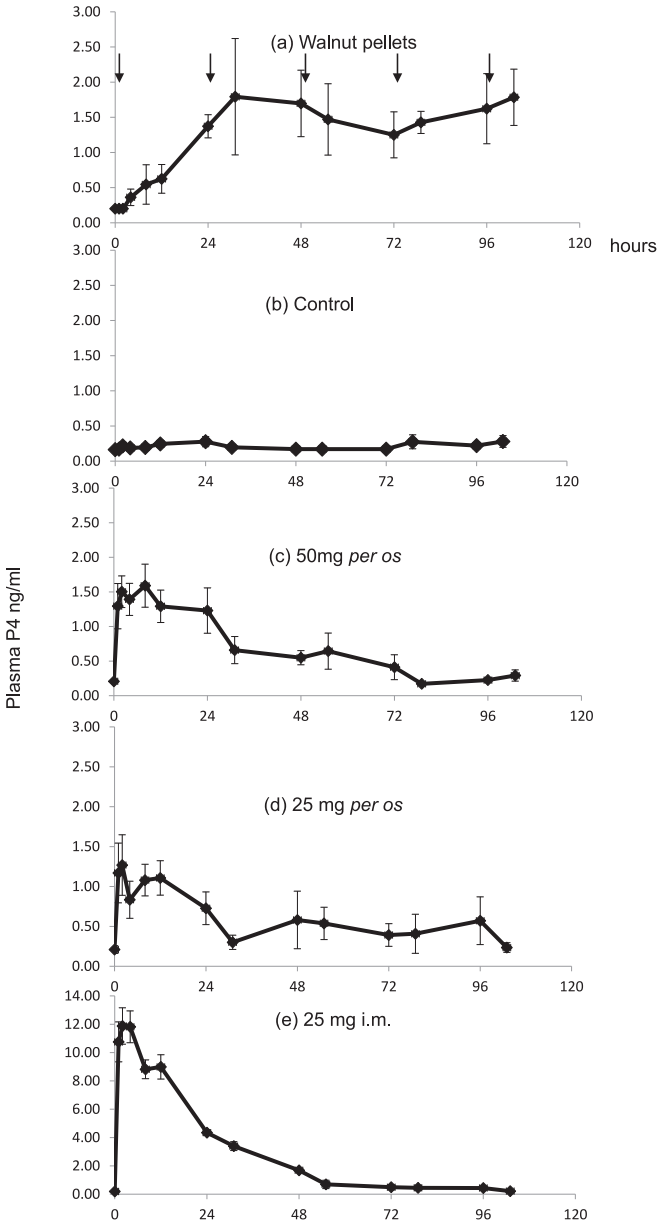

Fig. 3. Plasma progesterone (P4) concentrations (mean ± SEM) in 16 ovary-intact anoestrus goats in a Latin square experiment where goats were (a) fed walnut pellets orally every morning over 5 consecutive days (250 g/animal), (b) orally supplemented with 5 mL of sesame oil as the control group, (c) orally supplemented with 50 mg of P4 in 5 mL of sesame oil, (d) orally supplemented with 25 mg of P4 in 5 mL of sesame oil, and (e) intramuscularly injected once with 25 mg of P4 in 5 mL of sesame oil. Time 0 is 1 h before distribution or injection. Arrows indicate the time of pellet distribution.

Distribution of walnut pellets every morning and afternoon over 2.5 days in ovariectomised goats (Experiment 3) induced a rapid increase in P4 blood plasma concentration to > 0.5 ng/mL as soon as 2 h after the first distribution of pellets, and later reached 2.05 ng/mL at 48 h. The P4 concentration was maintained between 1.00 and 2.00 ng/mL 12–72 h after the first distribution (Fig. 5a). Plasma concentrations of P4 in goats receiving walnut pellets remained elevated after distribution was completed (48 h) but decreased slowly to reach 0.50 ng/mL 96 h after the start of distribution. As expected, the kinetics of plasma concentrations in the walnut group goats differed from those of the control group goats, which remained < 0.27 ng/mL during the whole experiment (Fig. 5b). Goats receiving 100 mg of P4 orally also showed a very rapid increase of P4 blood plasma concentration to > 0.5 ng/mL as soon as 1 h after oral administration, peaking at 2.77 ng/mL 8 h after administration and remaining around 0.50 ng/mL at 72 h (Fig. 5c).

Offering 1 250 g of walnut pellets during a single day to ovariectomised goats (Experiment 4) clearly exceeded the feeding capacity of most goats: 10 of 12 goats ate > 600 g but only three ate close to 1 250 g. On average, walnut pellet intake was 0.833 ± 0.096 g/head (range 125–1 250 g; Fig. 6a). Eating these walnut pellets induced a rapid increase in P4 blood plasma concentration in all goats to > 0.5 ng/mL as soon as 1 h after the first distribution, reaching 1.91 ng/mL 24 h after the first distribution (Fig. 6b). Plasma concentrations of P4 remained elevated after the end of the pellet distribution (12 h) and decreased slowly to reach 0.41 ng/mL 72 h after the last distribution. The evolution of the plasma concentrations in goats in the walnut group differed from that of goats in the control group, which remained < 0.12 ng/mL during the whole experiment (Fig. 6c). As before, goats receiving 100 mg of P4 orally also showed a very rapid increase of P4 blood plasma concentrations to > 0.5 ng/mL as soon as 1 h after oral administration, peaking at 4.81 ng/mL 1 h after administration and decreasing slowly to < 0.50 ng/mL at 56 h (Fig. 6d).

In Experiments 5–7, we limited the daily offer of walnut pellets to 600 g/head over either 1 or 3 days. After 1 day, blood plasma P4 rose to 2.42 ng/mL 24 h postdistribution and decreased to < 0.50 ng/mL as soon as 72 h afterwards (Supplementary Fig. S1). After 3 days, blood plasma P4 was maintained at 1.70 ng/mL 24–72 h after the first distribution (Supplementary Fig. S1) or was maintained between 0.94 and 1.04 ng/mL 24–72 h after the first distribution (Fig. 7). Blood plasma concentrations of P4 in goats of the two control groups (FGA and control) remained < 0.50 ng/mL in all samples (Fig. 7 and Supplementary Fig. S1).

Clinical efficacy

Clinical efficacy was tested only in the final Experiment 7 (Fig. 7). All goats of the three groups ovulated. The group of goats receiving walnut pellets showed the same percentages of goats showing oestrous behaviour at their first ovulation, of goats ovulating, and of goats experiencing short ovulatory cycles as the group of goats receiving no treatment (Table 5). These two parameters differed significantly from those of the FGA-treated goats (Table 5). In sum, the distribution of walnut pellets was unable to induce oestrous behaviour in all goats at their first ovulation and to restore normal functioning of the *corpus luteum* in all goats.

Discussion

The major result from our study was the very high content of P4 and its metabolites in walnut leaves of *Juglans regia* specifically. A high P4 content was previously described by Pauli et al. (2010), but in comparison, the highest concentration measured in our *Juglans regia* leaf samples (287 mg/kg of DM measured by EIA) is roughly 25 times higher. Using the GC–MS/MS assay, we also found a high P4 concentration of ~15 mg/kg in walnut leaves of several *Juglans regia* varieties, and high concentrations of many P4 metabolites such as 5α-, 5β-, 20α- and 20β-reduced metabolites of P4 in the range 1–11.5 mg/kg. The two methods of detection (EIA vs GC–MS/MS) gave results obviously not of the same order. This difference could be attributed to the methods themselves and to the specificity of the EIA antibody, which may also detect compounds related to P4. We detected very high concentrations of β-sitosterol and three other phytosterols, which may be precursors of P4 within the plant or in animals to which walnut leaf pellets were distributed. Such high concentrations of P4 in plants have not been

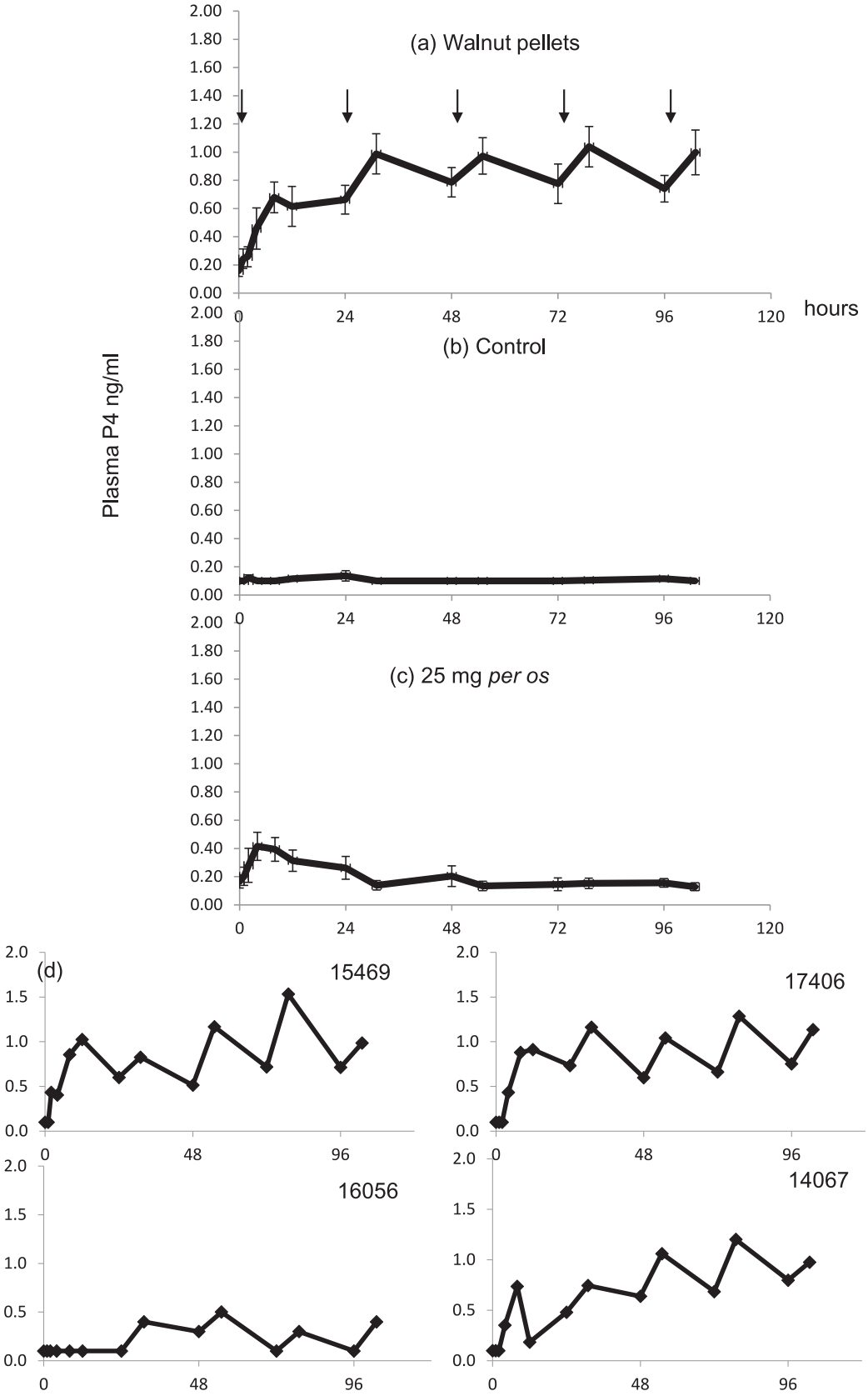

Fig. 4. Plasma progesterone (P4) concentrations (mean ± SEM) in 11 ovariectomised goats in a Latin square experiment orally receiving (a) 250 g/animal of walnut feed pellets over 5 days, (b) 5 mL of sesame oil as the control group, (c) 25 mg of P4 in 5 mL of sesame oil. Time 0 is 1 h before the start of pellet distribution. Arrows indicate the time of pellet distribution. Individual examples of goats receiving walnut pellets are shown in Fig. 4d.

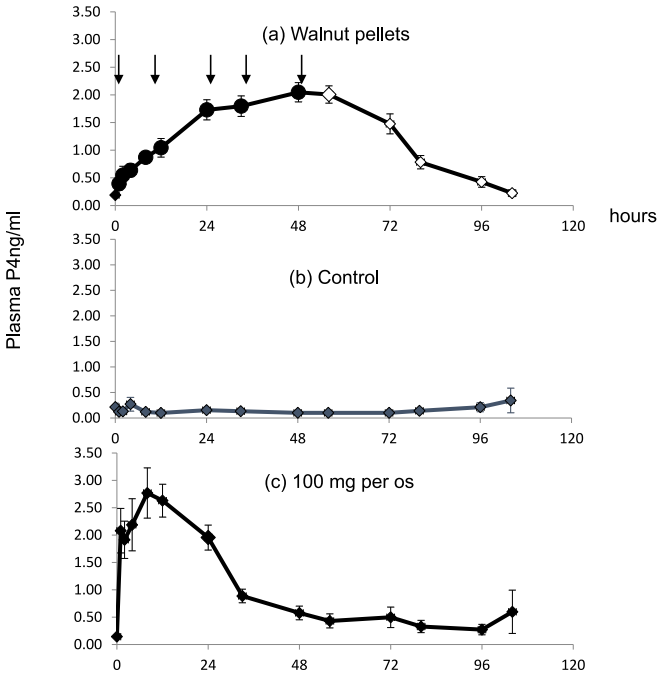

Fig. 5. Plasma progesterone (P4) concentrations (mean ± SEM) in eight ovariectomised goats in a Latin square experiment orally receiving (a) 250 g/animal twice daily of walnut feed pellets over 2.5 days (open dots are after the end distributions), (b) 5 mL of sesame oil as the control group, (c) 100 mg of P4 in 5 mL of sesame oil. Time 0 is 1 h before the start of distribution. Arrows indicate the times of pellet distribution.

described elsewhere except in 'benalu duku' leaves (Lazuardi and Hermanto, 2016, Lazuardi et al., 2019); but our analysis of that leaf material collected in Vietnam did not detect significant amounts of P4 (Supplementary Material S1).

By using a simple and inexpensive technique of P4 extraction with ethanol followed by a P4 EIA, we were able to show huge phenotypic variability of P4 concentration within *Juglans regia* leaves. Among the 182 varieties sampled, the range was 35–287 mg/kg of DM, and a continuum between these two extremes within the species is evident. The other *Juglans* species that we tested (*J. nigra*, *J. mollis* and *J. major*) did not contain P4 above the limit of detection of the assay (0.25 mg/kg of DM) or were very close to it. Moreover, high P4 content was only detectable in leaves but not in any other part of the plant. Flowers, fruits, oil, cake and husk did not contain P4 in significant concentrations, which shows that its presence may be linked to the specific metabolism of leaves.

The detection of P4 in plants and the effects of P4 on plant physiology have been extensively studied. A review on steroidogenesis in plants reported the presence of P4, the enzymes of steroidogenesis, and P4 membrane receptors in many plants (Lindemann, 2015). P4 content was shown to vary between plant tissues and organs, but the concentrations reported were substantially lower than those reported in the current study. Lindemann (2015) suggested that P4 may be involved in different components of plant development: cell division, root and shoot growth, embryo growth, flowering, pollen tube growth and callus proliferation, and that progesterone is part of a stress factor response in wheat. Our results showing that only walnut leaves of *Juglans regia* contain large concentrations of P4 suggest a qualitatively different hypothesis: (1) this trait has been selected relatively recently within the species because other *Juglans* species did not contain P4, and (2) it may be linked to a specific trait of the leaves themselves. A putative role in the resistance to pests such as aphids or diseases transmitted by aphids, which can be specific to leaves, is an interesting

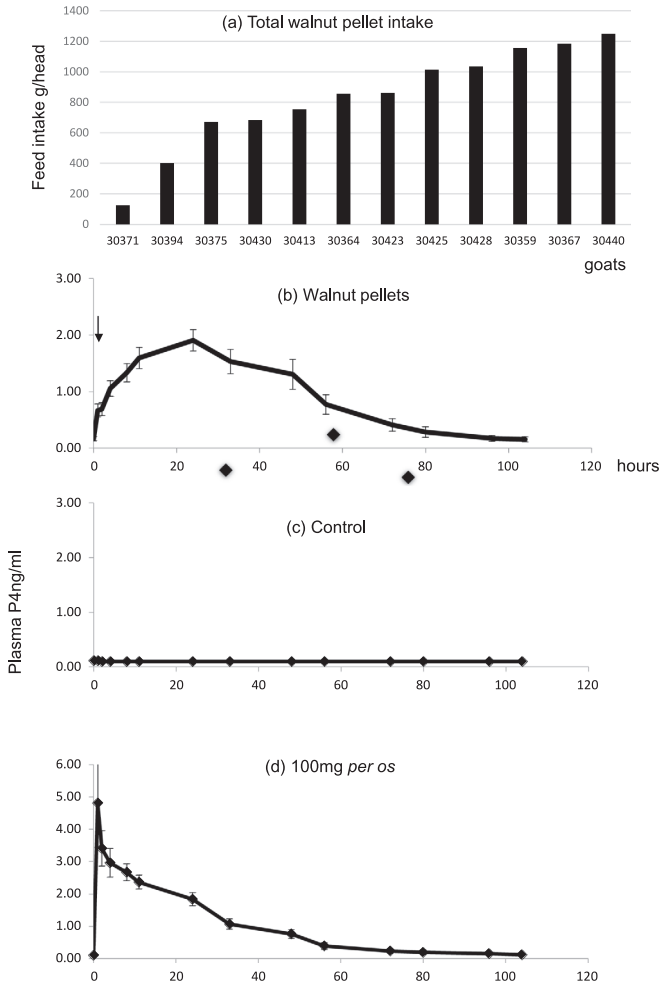

Fig. 6. Feed intake (a) and plasma progesterone (P4) concentrations (mean ± SEM) (b) in 12 ovariectomised goats in a Latin square experiment in which up to 1 250 g of walnut pellets was offered in a single day, compared with (c) goats orally receiving 5 mL of sesame oil as the control group, or (d) goats orally receiving 100 mg of P4 in 5 mL of sesame oil. Time 0 is 1 h before the start of distribution. The arrow indicates the time of pellet distribution.

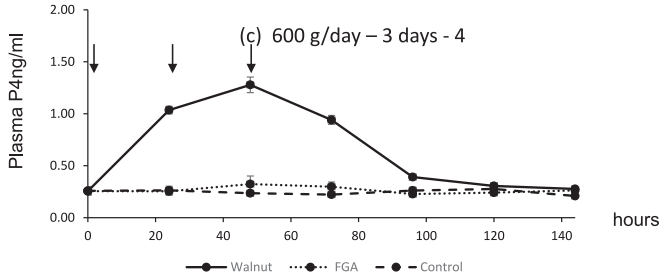

Fig. 7. Plasma progesterone (P4) concentrations (mean ± SEM) in 14 ovary-intact anoestrus goats orally receiving 600 g of walnut feed pellets every morning (black dot, continuous lines) over 3 days ending 4 days before the introduction of males, compared with 12 equivalent goats receiving control pellets and left untreated, and compared with 12 goats treated with an intravaginal sponge containing fluorogestone acetate (FGA). Time 0 is 1 h before the start of distribution. Arrows indicate the times of pellet distribution.

hypothesis. Large differences have been shown to exist in five walnut cultivars relating to the reproductive efficiency of the dusky-veined aphid (*Panaphis juglandis*), which is a major pest in walnut production (Akkopru et al., 2015). Much remains to be investigated regarding this unexpectedly high P4 content in *Juglans regia* walnut

Table 5
Number of goats, number of non-cyclic goats, percentages of goats showing oestrous behaviour at their first ovulation, percentages of goats ovulating, and percentages of goats experiencing short ovulatory cycles. Goats received an FGA intravaginal sponge for 10 days (FGA) where the sponge was removed the day males were introduced; walnut pellets fed for 3 days (Walnut), 6 to 4 days before introduction of males; or goats had no treatment (Control). Light exposed- and melatonin-treated males were introduced in April.

Group	Total number of goats	Number of non-cyclic goats	Oestrus at first ovulation (%)	Goats ovulating (%)	Short cycles (%)
Control	12	10	80[a]	100	90[ad]
FGA	12	12	100[ab]	100	0[e]
Walnut	14	13	54[ac]	100	77[ad]

Statistical analysis: First ovulation. a = not significant; b vs c, $P < 0.01$.
Short cycles. a = not significant; d vs e, $P < 0.001$.
FGA = fluorogestone acetate.

leaves, especially its genetic control and its role in plant physiology, for which we recently started a programme using the same tree collection described for this work.

A high P4 concentration led us to try using walnut leaves to produce feed pellets, which could be distributed to goats to replace the first phase of the hormonal treatment and which is often used to control ovulation and oestrous in goats and sheep. We have described a manufacturing process resulting in feed pellets containing 32% of dry leaves. These pellets are palatable and can be distributed safely and with a good acceptance to goats, which can typically eat 600 g of pellets (192 g of dry leaves) per animal/-day over 3 consecutive days, without any refusal and without any apparent behavioural or health disorder. We demonstrated that eating these pellets produces a substantial increase in plasma P4 concentration, which begins within 1 h after distribution and persists as long as the pellets are distributed to goats. Plasma P4 concentrations measured during pellet distributions range between 1 and 2 ng/mL and are much higher than the concentrations observed during the short cycle induced by the male effect in goats (Chemineau et al., 1984). This plasma concentration is believed to induce oestrous behaviour and normal *corpus luteum* at the following ovulation, around 8 days after male introduction. Thus, we were expecting that the long duration ($\geq$ 48 h) and high P4 concentrations (1–2 ng/mL) in the plasma of goats would be sufficient to obtain similar percentages of oestrous behaviour and of normal *corpus luteum* as in goats previously treated by an intravaginal sponge containing FGA. However, the percentage of goats showing oestrous behaviour and the duration of *corpus luteum* for the goats of the walnut group did not differ significantly from those of the control group, indicating that our treatment was not effective in controlling these two reproductive parameters.

We propose two hypotheses to explain this result. The first is that despite the sustainable increase of P4 concentrations in plasma that we observed, the doses distributed to goats were insufficient, such that P4 did not finally reach its required target level in the central nervous system for oestrous behaviour and in the uterus for suppressing the short cycle. This hypothesis seems plausible for the uterus because of retrocirculation from the ovary to the uterus in normal metabolism. However, it seems less plausible for the central nervous system because we collected the blood from the jugular vein coming directly from the central nervous system, and we detected higher P4 concentrations than during the short ovulatory cycle after the male effect. It may also mean that our assertion that the P4 increased during the short cycle after the male effect was incorrect in that it was insufficient to re-establish oestrous behaviour and normal cycles at the second ovulation. We know that an intramuscular injection of 25 mg of P4 is sufficient to re-establish oestrous behaviour and normal cycles, and we can see from Experiment 1 that P4 plasma concentration reached 12 ng/mL and stayed > 4 ng/mL over 24 h (Fig. 3e).

The second hypothesis is that the antibody used in our P4 immunoassay to measure plasma P4 was not specific enough, also binding to other steroids with a chemical structure close to that of P4. The different tests of specificity described in the Material and methods section, which tested a broad range of P4-associated molecules, showed that our antibody cross-reacted with three P4 metabolites, 5α-dihydroprogesterone, isopregnanonole, and 5β-dihydroprogesterone. The first two were detected by GC–MS/MS in significant concentrations in walnut leaves. Moreover, P4 is metabolised/catabolised by the passage through the gastrointestinal tract of monogastrics (rodents, humans and pigs), which contains 5α-, 5β- and 20α-reductases (Adlercreutz and Martin, 1980, De Lignières et al., 1995). It is possible that the EIA assay measured P4 and P4 metabolites that do not act on P4 receptors, even though the first, 5α-dihydroprogesterone, also acts on the P4 nuclear receptor. Importantly, to our knowledge, P4 metabolism has not been explored in detail in ruminants compared with monogastrics; the rumen may or may not behave as a buffer compartment able to retain P4 before passing it into general circulation.

Further experiments are in progress concerning the potential effects of P4 in walnut leaves on the reproductive biology of goats. The first step is to try to increase the doses of walnut leaves given orally to goats, using a more direct consumption of leaves from the varieties of *Juglans regia* containing the highest concentrations of P4. The second step is to change the way and mode of walnut leaf product delivery to allow P4 to reach the uterus more easily and to decrease the effect of the first hepatic pass when walnut pellets are distributed orally.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2024.101392.

Ethics approval

All procedures were performed following European directive 2010/63/EU on the protection of animals used for scientific purposes and approved by the local ethics committee for animal experimentation (CEEA VdL, Tours, France, Numbers 2021040111221610 and 2021051909002944), especially regarding the 3Rs (Refinement, Reduction, Replacement). All experimental animals were provided by UEPAO (https://uepao.val-de-loire.hub.inrae.fr/).

Data and model availability statement

None of the data were deposited in an official repository. The data/models that support the study findings are available to reviewers, or available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

Author ORCIDs

P. Chemineau: https://orcid.org/0000-0002-1584-7285.
A.L. Lainé: https://orcid.org/0000-0002-0531-4944.
D. Gennetay: no ORCID number.
C. Porte: https://orcid.org/0000-0002-8356-4973.
D. Chesneau: no ORCID number.
C. Laclie: no ORCID number.
G. Goudet: https://orcid.org/0000-0001-7805-6985.
M. Meunier: https://orcid.org/0000-0002-0803-0322.
P. Lière: https://orcid.org/0000-0001-9603-0586.
A. Pianos: https://orcid.org/0000-0001-5417-5429.
A. Bernard: https://orcid.org/0000-0003-1334-3072.
E. Dirlewanger: https://orcid.org/0000-0003-3244-5197.
J.A. Delgadillo: https://orcid.org/0000-0001-8351-0660.
M. Keller: https://orcid.org/0000-0002-5445-7431.

CRediT authorship contribution statement

P. Chemineau: Writing – review & editing, Writing – original draft, Resources, Methodology. **A.L. Lainé:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **D. Gennetay:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **C. Porte:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **D. Chesneau:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **C. Laclie:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **G. Goudet:** Writing – review & editing, Writing – original draft, Resources, Methodology. **M. Meunier:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **M. Delmas:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **M.L. Greil:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **P. Liere:** Writing – review & editing, Writing – original draft, Resources, Methodology. **A. Pianos:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **A. Bernard:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **E. Dirlewanger:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **J.A. Delgadillo:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **M. Keller:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of interest

P. Chemineau, D. Chesneau, AL Lainé, C. Porte, D. Gennetay, ML Greil, M. Delmas are inventors of patent FR 3116720 “ Progesterone vegetale et utilisations » granted to INRAE, CNRS, Université de Tours and IFCE.

Acknowledgements

We thank Drs Hoa Nguyen, H. Cuong, Pham Kim Dang and Samira Sarter for collecting and sending ‘benalu duku’ leaves from Vietnam, which provided the results presented in Supplementary Material S1. We thank Olivier Lasserre and colleagues of the experimental unit (UEPAO) in Nouzilly for taking care of the animals during the study. We also thank Morgane Brachet, Timothée Zidat, Hélène Vacher and Adrien Acquistapace of UMR-PRC for their help during animal sampling and observation. We are grateful to Pascal Laurent, Daniel Vergneau, Jean-Yves Chevrollier and all technicians of the Unité Expérimentale Arboricole for their help during the collections of the walnut leaves and all technicians of the Unité Expérimentale Physiologie et Phénotypage des Porcs in St. Gilles for their help in processing the experimental pellets. The authors are also grateful to the whole hormonal assay platform in Nouzilly, France for undertaking the hormonal determinations. We thank Pierre Martin from CAPGENES for his enduring support of the programme.

Financial support statement

This work was supported by the Centre Val de Loire regional authority under CAPRINOIX Contract Numbers 85-C4646 and 2020-00138613, by the programme cadre CNE “Maîtrise de la reproduction des petits ruminants” Number C18042/32001390 and by INRAE-PHASE specific funding. Phytosterol assays were supported by the INRAE grant Vegetruies of the METABIO programme Number C12603.

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