Animal 20 (2026) 101779

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Progestogen priming is not essential to trigger ovulation in seasonal anoestrus ewes injected twice with a synthetic kisspeptin analogue

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ARTICLE INFO

Article history:
Received 1 October 2025
Revised 3 February 2026
Accepted 5 February 2026
Available online 12 February 2026

Keywords:
Follicle–stimulating hormone
Luteal phase
Luteinising hormone
Reproduction management
Small ruminants

ABSTRACT

A key aspect of husbandry is reproduction management, and current methods often rely on the use of hormonal treatments. In the ewe, which is a seasonal breeder, a combination of progestogen and equine Chorionic Gonadotropin is the benchmark method for reproduction control. However, for different reasons, these hormones are leading to an increase in societal aversion. In order to find an alternative to their use, we have designed a synthetic analogue (**C6**) of kisspeptin, a key neurotransmitter in reproduction control. In the present study, we evaluated whether C6 could be used to replace hormones to induce ovulation and a luteal phase in ewes during the non-breeding season. Previous knowledge indicates that a single injection of C6 is unable to trigger ovulation unless preceded by progesterone priming; therefore, we tested a combination of two injections. We spaced injections at 24, 36 or 48 h. For the first injection, we used either 2.5 or 15 nmol, whereas the dose of the second injection was 15 nmol for all experimental groups. The combination of 15+15 nmol doses injected 48 h apart proved the most efficacious. It induced ovulation and a luteal phase in more than 60% of treated ewes as established by progesterone blood concentration. To assess if a specific hormonal footprint could be associated with ewes that ovulated, we analysed the time course of LH, FSH and estradiol plasma concentrations. However, no specific hormonal signature indicative of treatment success could be pinpointed. This study shows that it is possible to induce ovulation and a luteal phase in ewes during the non-breeding season by replacing hormonal treatment with a double injection of C6. Nonetheless, further optimisation of the method is required to reach the efficacy of benchmark treatment.

Implications

Current methods to trigger ovulation in ewes require the use of two hormones: a progestagen derivative and the equine Chorionic Gonadotropin. These hormones are suspected of being neuroendocrine disruptors or posing problems to animal welfare. The present work shows that using two injections of a synthetic molecule derived from an endogenous neuromodulator acting in the brain it is possible to trigger ovulation. The use of this molecule might represent a possible alternative to overcome issues raised by current treatments, but the protocol requires further optimisation to be fully satisfactory.

Introduction

The central role of the neuropeptide kisspeptin (**Kp**) and its receptor (**KISS1R**) in reproductive physiology is widely accepted. Based on this tenet, the worth of pharmacological activation of KISS1R to manage livestock reproduction was tested with encouraging results. These results point to a potential alternative to current hormone treatments (i.e. progestogen and equine Chorionic Gonadotropin (**eCG**)) (Decourt et al., 2016; Decourt et al., 2019; Fanelli et al., 2022; Leonardi et al., 2020). In the present work, we used the Kp analogue C6 (Decourt et al., 2016) to test a new protocol for inducing ovulation in the ewe during the non-breeding season in the absence of hormonal treatment.

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https://doi.org/10.1016/j.animal.2026.101779

Since the discovery of the role of the Kp system in reproduction, the effect of its pharmacological modulation has been probed under physiological and pathological conditions in both humans and livestock. Even though four Kp isoforms have been described (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001), the majority of the studies were performed with only two of them: **Kp10** and **Kp54** (10 and 54 amino acids long, respectively). Human studies mainly focused on women and evaluated the effect of Kp54. Under different clinical protocols, Kp54 was able to lessen symptoms of reproductive disorders such as hypothalamic amenorrhoea (Jayasena et al., 2014b) and facilitate maturation of oocytes in women undergoing *in vitro* fertilisation therapy (Jayasena et al., 2014a).

The synthesis of a long peptide such as Kp54 is, however, a complex task incurring a significant cost. While such a cost is acceptable for human applications, it is a concern for livestock treatments. This is one reason why research aimed at developing field applications for livestock chiefly used Kp10. This choice was also backed up by the consideration that Kp10 and Kp54 show quite similar potency and efficacy *in vitro* (Muir et al., 2001). The main drawback of Kp10 is its shorter half-life compared to Kp54 (Chan et al., 2011; d'Anglemont de Tassigny et al., 2017; Dhillo et al., 2005; Liu et al., 2013). Indeed, an intravenous perfusion of Kp10, lasting 48 h, is required to trigger ovulation in the ewe in the absence of hormonal treatment (Caraty et al., 2007). This result represents an important proof of concept. Nevertheless, protocols implying an intravenous perfusion are unsuitable for field applications. To tackle the problematically short half-life of Kp10 and facilitate the design of a protocol suitable for field applications, we developed a Kp10 analogue named C6. This molecule is resistant to degradation and has an improved potency (Decourt et al., 2016). An intramuscular bolus injection of 15 nmol/ewe of C6 produced an increase of LH in the blood lasting up to 12 h. Under the same conditions, Kp10 is unable to increase LH plasma levels (Decourt et al., 2016). A subsequent study using C6 in goats showed its capacity to stimulate LH and FSH secretion and to induce ovulations both in the breeding and non-breeding seasons in does primed with a progestogen (Decourt et al., 2019). To check whether, by further increasing the duration of KISS1R stimulation, progestogen priming could be omitted, we designed a slow–release formulation containing C6. The use of this C6 containing formulation induced ovulation in 50% of treated ewes (Salzano et al., 2022). Despite these encouraging results, we had only partially overcame the issue of efficacy, suggesting that the duration of action is not the only key factor.

During the non-breeding season, ewes develop follicles in waves that degenerate if not sustained by an appropriate hormonal environment. Progestogen priming favours FSH over LH secretion by decreasing GnRH pulse frequency (Wildt et al., 1981). Increased FSH blood concentration supports follicular maturation, promoting the transition to the terminal phase of follicle development. We speculated that the sub-optimal ovulation rate obtained with the formulation might be caused by an inappropriate transition to the terminal phase of follicle development (Salzano et al., 2022).

Based on these premises, we thought that an initial injection of C6, by increasing FSH level, could assist the transition of the follicle to the terminal phase and a second injection would trigger ovulation by inducing an LH surge. To test this hypothesis, we injected the ewes twice with C6 during the non-breeding season in the absence of progestogen priming and eCG induction. To check for the best combination of time interval between injections and combination of doses, we performed two injections separated by 24, 36 or 48 h, combining different doses of C6. The goals were(i) to check if we could induce ovulation and a luteal phase in the absence of progestogen priming during the non-breeding season; (ii) to establish a possible correlation between the hormonal profile induced by the treatment and the occurrence of a luteal phase; (iii) to evaluate if the first injection would have an effect on the hormonal profile triggered by the second injection (desensitisation or priming).

Material and methods

Animal models

Experiments were carried out according to national and international regulations (Directive 2010/63/UE). All procedures were approved by the local Animal Ethics Committee (Comité d'Ethique en Expérimentation Animale Val de Loire) and authorised by the French Minister "de l'Education National, de l'Enseignement Superieure et de la Recherche" (authorisation number 01873.03 and APAFIS#10958-2017062714349167v7). Animals were housed at the INRAE research center facilities (Unité Expérimentale PAO, INRAE. Animal Physiology Experimental Facility, authorisation F371752 of the French Minister "de l' Agriculture").

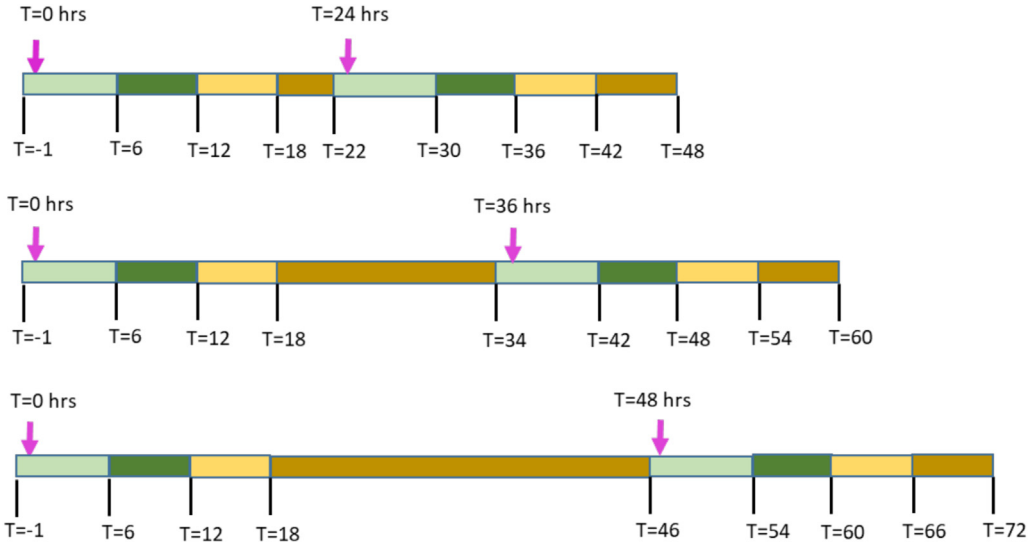
Experiments were performed during the non-breeding season in intact ewes of Ile-de-France breed (3–5 years old, weighing 60–90 kg) maintained under normal husbandry. To check that ewes were in seasonal anoestrus starting from February up to the end of March, a blood sample was collected twice weekly by venipuncture of the jugular vein. Samples were centrifuged, plasma collected and stored at −20 °C until processed to measure progesterone concentration. Animals showing progesterone concentrations below the detection limit of the assay for three consecutive weeks were considered in seasonal anoestrus.

Ewe preparation, drug administration and blood sampling

Three days before the experiment, ewes in seasonal anoestrus were placed in individual contiguous pens. On the day before the experiment, a catheter (ID 1.0, L 52 mm, Intraflon 2, Vygon, France) was inserted in the jugular vein to collect serial blood samples. Experimental setting is summarised in Fig. 1. To establish the basal blood concentration of hormones before treatment, 24 h after insertion of the catheter, three blood samples (2 mL each) were collected at 30-min intervals. All experimental groups (nine groups with N = 8 per group) received a first intramuscular injection of C6 (2.5 or 15 nmol/ewe in 1 mL saline solution) or saline solution. A second dose of C6 (15 nmol/ewe) or saline (for the control group) was injected at 24, 36 or 48 h after the first one. Blood samples (2 mL) were taken at various time intervals postinjection. Blood sampling intervals were identical for the nine groups (six treated and three control groups) during the first 22 h postinjection. From the time of injection up to 6 h postinjection, one sample every 30 min, from 6 to 12 h one sample every hour, from 12 to 18 h one sample every 2 h and from 18 to 22 h one sample each 4 h. For groups receiving a second injection at 24 h, the protocol above was repeated identically after the second injection. For groups receiving a second injection at 36- or 48-h intervals starting from 18 h after the first injection, blood samples were collected every 4 h up to 34 and 46 h, respectively. Then, the same protocol used after the first injection was applied. To check for ovulation, a blood sample was collected every day for progesterone assay starting from the day after the second injection and up to 14 days.

Hormone assays

Plasma LH and FSH concentrations were measured using previously described radioimmunoassays (Decourt et al., 2019; Montgomery et al., 1985; Pelletier et al., 1968). For the LH assay, 1055-CY-LH (equivalent to 2.2 NIH-LH-S1) was used as a standard. Intra-assay and inter-assay CV at 2.3 ng/mL were 5% and 9%,



	Time interval between treatment		
	24 hrs	36 hrs	48 hrs
Treatment	Saline+saline (N=8)	Saline+saline (N=8)	Saline+saline (N=8)
	2.5+15 nmol C6 (N=8)	2.5+15 nmol C6 (N=8)	2.5+15 nmol C6 (N=8)
	15+15 nmol C6 (N=8)	15+15 nmol C6 (N=8)	15+15 nmol C6 (N=8)

Fig. 1. Experimental scheme. The scheme illustrates sampling frequency (color code), time of drug or saline injections (arrows) and experimental groups used to test the capacity to induce ovulation in ewes during the non-breeding season. T = time (in hours). C6 Synthetic kisspeptin analogue.

respectively; assay sensitivity was 0.2 ng/mL. Plasma FSH levels were measured using the antibody oFSH (NIH) AFP-C5288113 and the oFSH ^{125}I (NIH) AFP-4117A iodinated in–house. The intra-assay and inter-assay CV at 1.2 ng/mL were 6% and 11%, respectively; assay sensitivity was 0.1 ng/mL. Estradiol assay (**E2**) required to be set up. E2 was extracted from the plasma using diethylether and dried at 37 °C. Given the expected low concentration of estradiol present in the blood of anestrous females, the maximum volume of plasma left after measuring the other hormones was extracted. All samples were reconstituted in 100 µl of phosphate buffer saline 0.1 M containing 1% of gelatine (**PBSg**). The percentage of recovery was evaluated by adding to some samples 50 µl of [2,4,6,16,17]^{3}H estradiol-17β (0.032 µCi/mL; NET317250UC, Revvity) prepared in PBSg. Average recovery was 74,4%.

E2 was quantified using a commercially available ^{125}Iodine-E2 radioimmunoassay kit (DSL-4800, Ultra-sensitive Estradiol RIA, Beckman Coulter). The kit was validated using pools of plasma obtained from ewes during the breeding season. To test for parallelism, these pools were serially diluted after extraction and compared with the standard curve of the assay. The percentages of tracer bound (B/B0) from serially diluted samples were parallel to the standard curves in the range of quantities used for the final assay, confirming the specificity of the assay. By adding known amounts of steroids to samples, we demonstrated that these assays accurately and reliably quantify E2. The cross-reactivity for the E2 antiserum, as reported by the manufacturer, is 2.56% for 17β-estradiol-3-glucuronide, 2.4% for estrone and < 1% for other oestrogens or oestrogenic metabolites.

Experimental samples were run in duplicate (n = 176) unless less than 500 µl of plasma was available, in which case they were run as singletons (n = 16) following the manufacturer's recommendations with minor modifications. Briefly, we adapted the standard curve, which ranged between 0.078 and 11.25 pg/tube and the average sensitivity across experiments was 0.02 pg/tube, as determined by values with a B/B0 of 97%. Reconstituted samples (40 µl) were incubated at room temperature for 4 h with 100 µl of anti-E2 antiserum (diluted 1:3.5 in PBSg). Then, 100 µl of ^{125}I-E2 (diluted 1:3, in PBSg) was added, and tubes were incubated at 4 °C for 24 h. The precipitating reagent (500 µl) was added and tubes were incubated for 20 min at room temperature, prior to a centrifugation step (3 000 g for 30 min, 4 °C). Finally, supernatants were decanted, and pellets were counted with a gamma counter. The amount of E2 present in each tube was obtained using a linear regression of the log–transformed concentrations of the standards and logit transformation of the B/B0. All samples were above the detection limit. Obtained values were then averaged between duplicates and corrected for the volume extracted and the recovery to obtain a concentration. Samples were assayed in different runs with internal controls, all samples from an animal being assayed in the same run. The mean intra- and inter-assay CV were respectively 9% and 11%.

Progesterone was measured by an ELISA assay on 96-well plates (Immuno Nunc Maxisorp C96) as previously described (Decourt et al., 2016). Briefly, plates were coated with a goat anti-mouse IgG (Uptima UP462140, Interchim). After washing plates were incubated with the secondary antibody (mouse monoclonal anti-progesterone, AbD, Serotec (10 µL in 16 mL Tris-BSA) together with 10 µL of the samples overnight at 4 °C. The following day, 50 µL/well of progesterone-alpha alkaline phosphatase conjugate (Immunometrics Ltd.) (10 µL P4-pal 6 mL Tris-BSA) was added to each well and incubated in the dark for 1 h. Plates were washed and then incubated with P-Nitrophenyl Phosphate (Sigma-Aldrich) for about 2 h at 37 °C, and absorbances were recorded at

405 nm with a plate reader (TECAN). Intra-assay coefficient of variation averaged 8.5%, and assay sensitivity was 0.25 ng/mL.

Data elaboration and statistical analysis of results

Ewes responding to treatment were identified using the following criteria: the progesterone level should be above 0.5 ng/mL for at least 9 consecutive days and the mean progesterone value of the consecutive days should be above 1 ng/mL. All ewes not respecting these criteria, even if they showed a progesterone increase, were considered non-responder.

Maximal amplitude of response and the area under the curve (**AUC**) for both LH and FSH were calculated by separating the experiment into two parts. The first part started with basal sampling before the first injection of C6 or saline and ended 12 h postinjection. The second part started with basal sampling before the second injection and terminated 12 h later. The choice of 12 h is related to the mean duration of a natural LH surge (Evans et al., 1997). To calculate maximal amplitude and AUC, basal was obtained by averaging the concentration of three samples before treatment and was subtracted to data obtained after treatment. The following criteria were used to identify an increase of LH and FSH: a maximal height after basal subtraction of at least 1 and 0.1 ng/mL, respectively, a minimum 10% of difference between minimum and maximum and at least three consecutive samples above basal. To be considered, a consequence of treatment peak maximum should be reached between 1 and 8 h after drug injection. Data not fitting these criteria were excluded from analysis.

AUC and maximal amplitude of first and second injection were compared for each individual to check for desensitisation or priming effect.

To check whether the difference in the hormonal profile induced by the second injection would predict responders vs non-responders, the AUC of LH and FSH induced by the second injection was compared. Because the number of responders was zero or low for some conditions, this comparison was not possible for all experimental groups. Data were not normally distributed; hence, individual maximal amplitude and AUC were analysed using the Wilcoxon matched-pairs signed rank test when comparing the effect of the first and second injection and the Mann-Whitney test when comparing AUC of responders and non-responders. For the Wilcoxon test, we indicate the W which is the sum of signed ranks. Results are presented as the median and lower and upper quartiles. All calculations were performed with the software GraphPad Prism 10.5.0, and the significance threshold was placed at alpha = 0.05.

Results

Control groups

In the control groups, regardless of the time interval between saline injections, there was no LH increase after treatment and consequently no difference between the first and the second injection. No ewes showed an increase of progesterone over the 0.5 ng/mL threshold (Fig. 2).

Combination of 2.5 and 15 nmol doses

Injections at a 24-h interval

Two out of eight ewes showed a progesterone increase, but none met the criteria to be considered a responder (Fig. 3A). LH maximum concentration was smaller after the first injection compared to the second injection (P = 0.02, W = 32, n = 8) (Fig. 3A and Table 1). Comparison of AUC showed that the second injection of

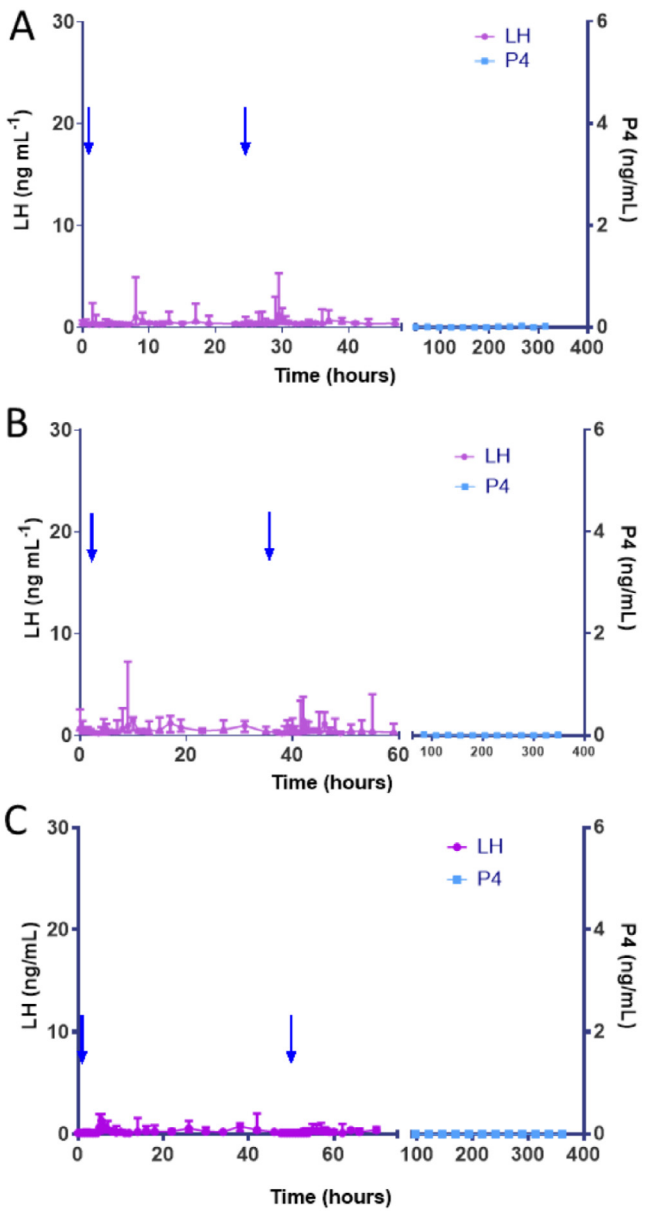


Fig. 2. LH and progesterone profiles induced by saline injections. Treatment of ewes with two saline injections (blue arrows) at 24 (A), 36 (B), or 48-h (C) intervals produced no effect on LH and progesterone plasma concentrations. Data are expressed as median and interquartile range.

C6 induced a significantly larger amount of LH release compared to the first one (P = 0.02, W = 32, n = 8) (Table 1). Following the first injection, after reaching the maximum, the FSH plasma concentration declined below the pretreatment level. However, it rose above pretreatment level before the second injection (Fig. 3A). The second injection triggered an FSH maximum larger than the first one (P = 0.02, W = 32, n = 8) (Table 2). Accordingly, the AUC was larger after the second injection compared to the first one (P = 0.01, W = 36, n = 8) (Table 2).

Injections at a 36-h interval

Four ewes showed an increase of progesterone, but none of them met the criteria for responders (Fig. 3B). The second injection induced a maximal amplitude larger than the first one, but the difference was not statistically significant (P = 0.46, W = 12, n = 8) (Fig. 3B and Table 1). Similarly, the total amount of LH released

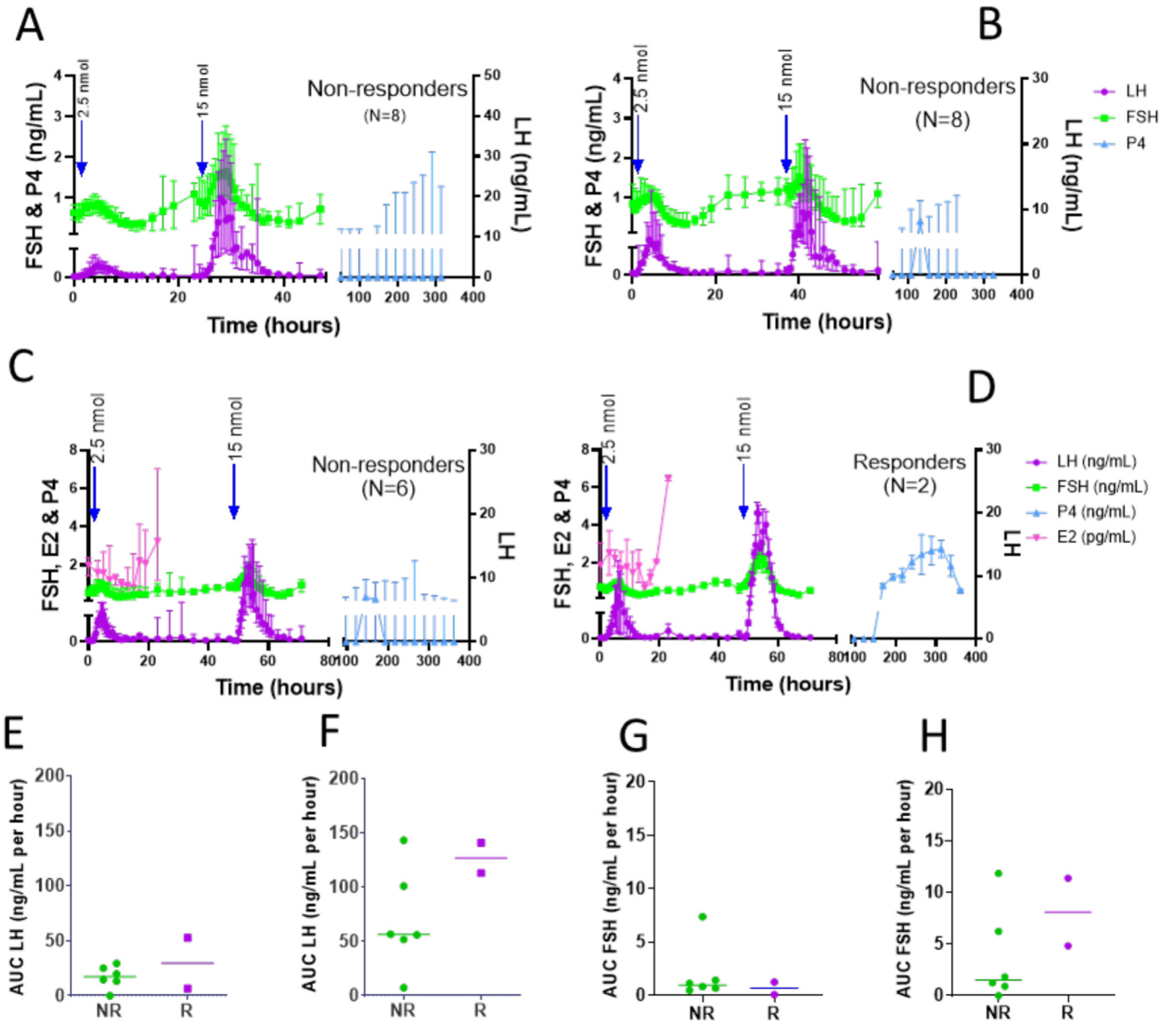


Fig. 3. Hormonal profiles induced by an injection of 2.5 nmol of the synthetic kisspetin analogue C6, followed 24, 36 or 48 h later by a second injection of 15 nmol. (A) LH, FSH, and progesterone plasma levels following C6 injections (blue arrows) 24 h apart. Both injections induced an increase in LH and FSH plasma levels, but no ewe showed a progesterone increase fitting the criteria for responder (i.e. progesterone level should be above 0.5 ng/mL for at least 9 consecutive days and the mean progesterone value of the consecutive days should be above 1 ng/mL). (B) LH, FSH, and progesterone plasma levels following C6 injections 36 h apart. Both injections induced an increase in LH and FSH plasma levels, but no ewe showed a progesterone increase fitting the criteria for responder. (C and D) LH, FSH, Estradiol, and progesterone plasma levels following C6 injections 48 h apart. Both injections induced an increase in LH and FSH plasma levels. Estradiol concentration increased after the first injection; no dosage was performed after the second injection. Six ewes did not meet the responders' criteria (C) whereas two did (D). (E–H) AUC of responders and non-responder after the first injection (E LH, and G FSH) and after the second injection (F LH, and H FSH) showed no difference. Data are expressed as median and interquartile range. NR non-responders, R responders, AUC area under the curve, C6 synthetic kisspeptin analogue, E2 Estradiol, P4 progesterone.

after the second injection was larger compared to the first injection but not statistically different ($P = 0.14$, W = 22, n = 8) (Table 1). First and second injection induced a similar maximal amplitude ($P = 0.31$, W = 16, n = 8) (Fig. 3B and Table 2) and resulted in a similar total amount of FSH released ($P = 0.15$, W = 22, n = 8) (Table 2).

Injections at a 48-h interval

After treatment, five out of eight ewes showed an increase of progesterone, but only two of them met the criteria for responders (Fig. 3C and D). Injections triggered an increase of LH in both non-responders and responders (Fig. 3C and D). Qualitative comparison of AUC of responders vs non-responders after the first (Fig. 3E) or the second (Fig. 3F) injection showed no clear difference, but due to the small number of responders (n = 2), it was not possible to perform a statistical analysis. When data from non-responders and responders were pooled together, the maximal amplitude was larger after the second injection ($P = 0.03$, W = 26, n = 7) (Table 1). Accordingly, AUC comparison showed that the second injection induced the release of a significantly larger amount of LH compared to the first injection ($P = 0.02$, W = 34, n = 8) (Table 1).

Injections triggered an increase of FSH in both non-responders and responders (Fig. 3C and D). When comparing AUC after the first (Fig. 3G) and second (Fig. 3H) injection of non-responders vs responders, no clear difference was visible, but due to the small

Table 1
Maximal amplitude (Max) and total amount of LH released (AUC) following C6 injections in ewes during the non-breeding season.

Time interval (hours)	Injection	LH			
		2.5+15 nmol C6		15+15 nmol C6	
		Max (ng/mL)	AUC (ng/mL·hour)	Max (ng/mL)	AUC (ng/mL·hour)
24	1st injection	3.0 (1.8; 5.9) (n = 8)	13.9 (4.9; 21.8) (n = 8)	20.8 (14.4; 24.4) (n = 8)	90.5 (67.5; 127.8) (n = 8)
24	2nd injection	15.0 (6.4; 33.6) (n = 8)	80.4 (16.05; 143.8) (n = 8)	11.1 (6.0; 16.1) (n = 8)	46.5 (20.8; 64.3) (n = 8)
36	1st injection	6.8 (4.2; 14.4) (n = 8)	28.0 (18.5; 56.0) (n = 8)	6.9 (4.1; 15.7) (n = 8)	25.0 (18.2; 54.5) (n = 8)
36	2nd injection	10.4 (5.8; 19.3) (n = 8)	55.5 (21.8; 106.0) (n = 8)	10.7 (6.0; 20.9) (n = 8)	58.5 (24.5; 100.0) (n = 8)
48	1st injection	6.1 (3.2; 6.3) (n = 7)	19.9 (13.3; 29.5) (n = 7)	18.2 (5.5; 21.0) (n = 8)	77.1 (21.5; 107.0) (n = 8)
48	2nd injection	15.7 (9.9; 20.0) (n = 7)	101.0 (56.1; 140.9) (n = 7)	9.3 (4.3; 25.3) (n = 8)	64.6 (25.1;129.0) (n = 8)

Abbreviations: Max = Maximal amplitude; AUC = Area Under the Curve; C6 = synthetic kisspeptin analogue.
All data are expressed as median, and lower and upper quartiles.

Table 2
Maximal amplitude (Max) and total amount of FSH released (AUC) following C6 injection in ewes during the non-breeding season.

Time interval (hours)	Injection	FSH			
		2.5+15 nmol C6		15+15 nmol C6	
		Max (ng/mL)	AUC (ng/mL·hour)	Max (ng/mL)	AUC (ng/mL·hour)
24	1st injection	0.3 (0.2; 0.4) (n = 8)	0.7 (0.5; 1.6) (n = 8)	1.1 (1.1; 1.8) (n = 8)	5.5 (2.9; 8.3) (n = 8)
24	2nd injection	0.8 (0.4; 2.1) (n = 8)	14.2 (9.1; 19.0) (n = 8)	1.3 (0.9; 1.6) (n = 8)	3.7 (1.4; 10.0) (n = 8)
36	1st injection	0.3 (0.3; 0.7) (n = 8)	0.7 (0.2; 1.5) (n = 8)	1.8 (1.2; 2.1) (n = 8)	8.1 (6.4; 11.2) (n = 8)
36	2nd injection	0.7 (0.4; 0.9) (n = 8)	1.5 (0.8; 309) (n = 8)	0.4 (0.1; 0.6) (n = 8)	0.7 (0.2; 2.6) (n = 8)
48	1st injection	0.4 (0.2; 0.5) (n = 8)	1.0 (0.5; 1.4) (n = 8)	0.7 (0.3; 1.0) (n = 8)	2.5 (0.5; 4.9) (n = 8)
48	2nd injection	0.7 (0.3; 2.0) (n = 8)	3.3 (1.0; 3.3) (n = 8)	0.47 (0.2; 1.3) (n = 8)	2.1 (0.6; 6.4) (n = 8)

Abbreviations: Max = Maximal amplitude; AUC = Area Under the Curve; C6 = synthetic kisspeptin analogue.
All data are expressed as median, and lower and upper quartiles.

number of responders (n = 2), it was not possible to perform a statistical analysis. When data from non-responders and responders were pooled together, the two injections showed a similar maximal amplitude (P = 0.15, W = 22, n = 8) (Table 2). The two C6 injections also produced similar total amounts of FSH release (P = 0.20, W = 20, n = 8) (Table 2). An increase in estradiol concentration started about 17 h after the first injection and reached a maximum at about 22 h. Due to the small number of responders (n = 2), it was not possible to perform a statistical analysis comparing non-responders and responders.

Combination of 15 and 15 nmol doses

Injections at a 24-h interval

Three out of eight ewes showed a progesterone increase meeting criteria for responders (Fig. 4A and B). Injections triggered an LH increase in both non-responders and responders (Fig. 4A and B). When comparing the AUC of the first (Fig. 4C) and second (Fig. 4D) injection of non-responders vs responders, no difference was observed (first injection P = 0.57, U = 5, second injection P = 0.99, U = 7). Pooling data from non-responders and responders showed that the first injection of C6 induced an LH peak reaching a larger maximal amplitude compared to the second injection (P = 0.02, W = –32, n = 8) (Table 1). Concurrently, the AUC of the first peak was large compared to that of the second peak (P = 0.0 2 W = –32, n = 8) (Table 1).

Injections triggered an FSH increase in both non-responders and responders (Fig. 4A and B). When comparing the AUC of the first (Fig. 4E) and second (Fig. 4F) injection of non-responders vs responders, no difference was observed (first injection P = 0.99, U = 7, second injection P = 0.38, U = 4). Pooling data from responders and non-responders showed that the first and second injec-

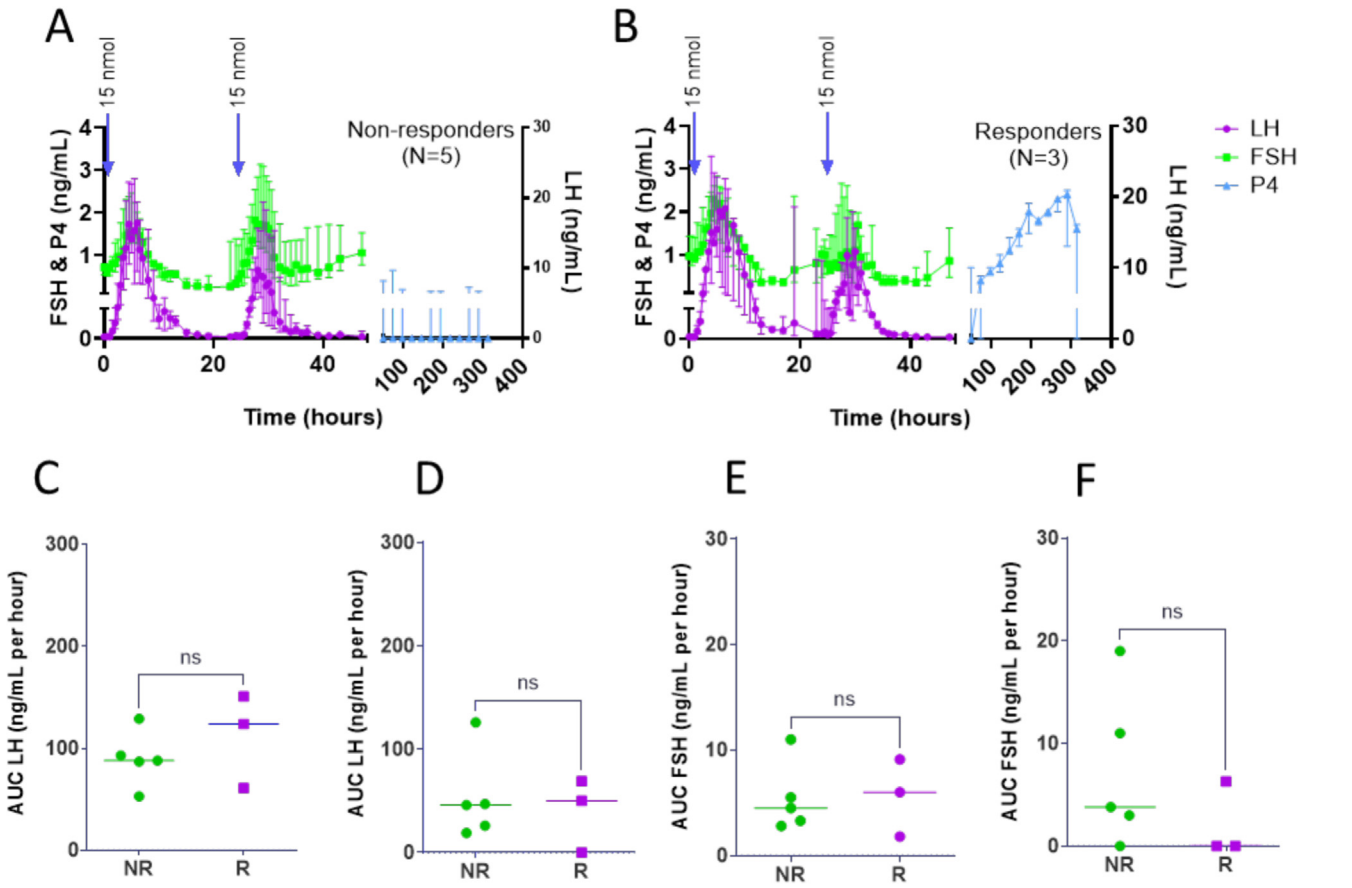


Fig. 4. Hormonal profiles induced by two injections of 15 nmol of C6 24 h apart. (A–B) LH, FSH, and progesterone plasma levels following two C6 injections (blue arrows) 24 h apart. Both injections induced an increase in LH and FSH plasma levels. Five ewes showed a progesterone increase not fitting the criteria for responder (A) (progesterone level should be above 0.5 ng/mL for a least 9 consecutive days and mean progesterone value of the consecutive days should be above 1 ng/mL) whereas 3 did (B). (C–F) AUC of responders and non-responder after the first injection (C LH, and E FSH) and after the second injection (D LH, and F FSH) showed no difference. Data are expressed as median and interquartile range. NR non-responders, R responders, ns not significant, AUC area under the curve, C6 synthetic kisspeptin analogue, P4 progesterone.

tions elicited a maximum increase of FSH that was similar (P = 0.64, W = –8, n = 8) (Table 2). Total amount of FSH released following the two injections was also similar (P = 1, W = 0, n = 8) (Table 2).

Injections at a 36-h interval

Six ewes out of eight showed a progesterone increase, but only two of them met the criteria for responders (Fig. 5A and B). Injections triggered an LH increase in both non-responders and responders (Fig. 5A and B). A qualitative examination of AUC of the first (Fig. 5C) and second (Fig. 5D) injection of non-responders vs responders suggests no difference between the two groups, but due to the small number of responders (n = 2) was not possible to perform a statistical analysis. Pooling data from non-responders and responders showed that the first and second injections elicited a similar maximum LH amplitude (P = 0.46, W = 12, n = 8) (Table 1). Total amount of LH released after the first and second injection was similar (P = 0.15, W = 22, n = 7) (Table 1). Injections triggered an FSH increase in both non-responders and responders (Fig. 5A and B). A qualitative examination of AUC of the first (Fig. 5E) and second (Fig. 5F) injection of non-responders vs responders suggests no difference, but due to the small number of responders (n = 2), it was not possible to perform a statistical analysis. Pooling data from non-responders and responders showed that first injection elicited a larger increase of FSH (P = 0.008, W = –36, n = 8) (Table 2). Accordingly, the total amount of FSH released was also larger after the first injection (P = 0.008, W = –36, n = 8) (Table 2).

Injections at a 48-h interval

All ewes but one showed an increase of progesterone, and five ewes met the criteria for responders (Fig. 6A and B). Injections triggered an LH increase in both non-responders and responders (Fig. 6A and B). When comparing AUC after the first (Fig. 6C) and second (Fig. 6D) injection of non-responders vs responders, no difference was observed (first injection P = 0.39, U = 4, second injection P = 0.12, U = 2). Pooling data from non-responders and responders showed that the first and second injection elicited a similar maximal increase (P = 0.84, W = –4, n = 8) (Table 2). Total amount of LH released was also similar for the two injections (P = 0.80, W = 4, n = 8) (Table 2).

Injections triggered an FSH increase in both non-responders and responders (Fig. 6A and B). When comparing the AUC of non-responders vs responders after the first (Fig. 6E) and second (Fig. 6F) injection, no difference was observed (first injection P = 0.99, U = 4.5, second injection P = 0.32, U = 4). Pooling data from non-responders and responders showed that maximal FSH amplitude was similar after the two injections (P = 0.92, W = –2, n = 8) (Table 2). The first and second injection also triggered a similar total amount of FSH release (P = 0.64, W = 8, n = 8) (Table 2). Estradiol levels were variable in the non-responders and responders groups with no clear trend.

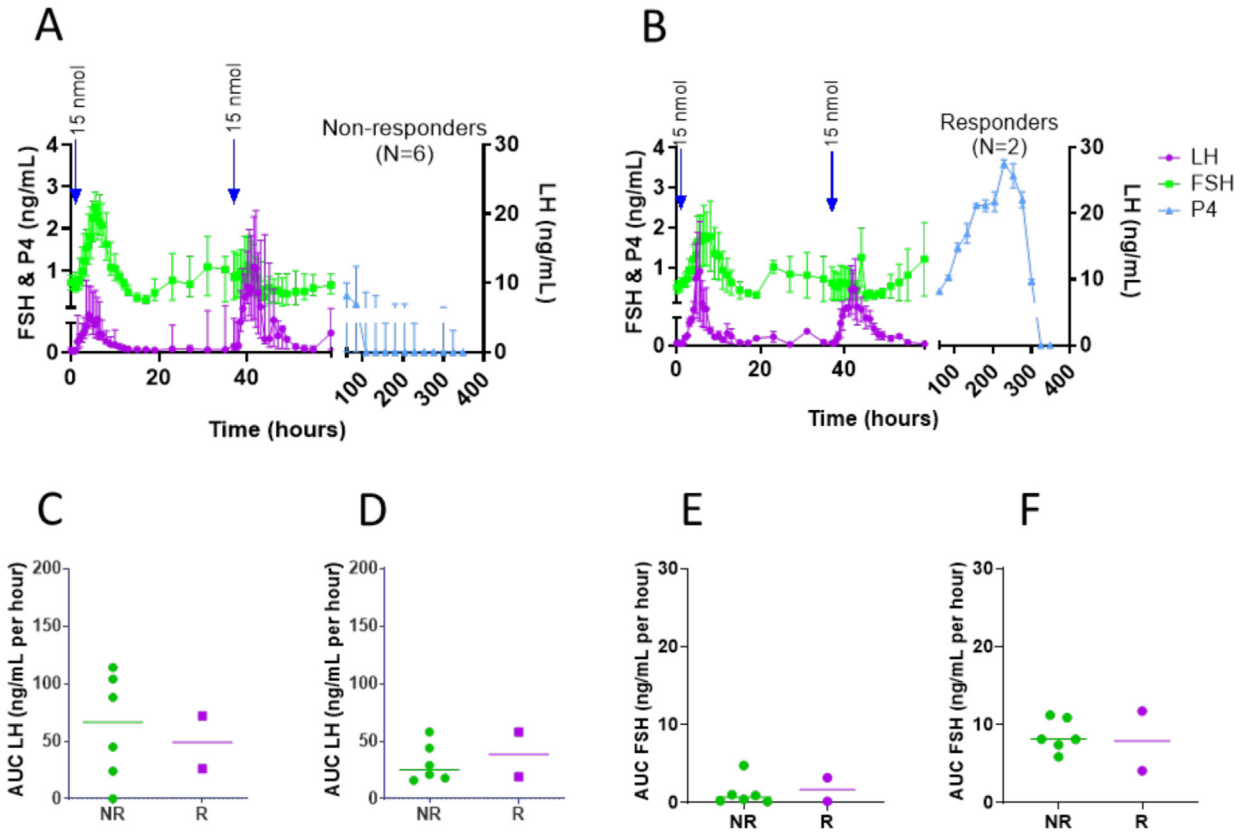


Fig. 5. Hormonal profiles induced by two injections of 15 nmol of C6 36 h apart. (A–B) LH, FSH, and progesterone plasma levels following two C6 injections (blue arrows) 36 h apart. Both injections induced an increase in LH and FSH plasma levels. Six ewes showed a progesterone increase not fitting the criteria for responder (A) (progesterone level should be above 0.5 ng/mL for a least 9 consecutive days and mean progesterone value of the consecutive days should be above 1 ng/mL) whereas 2 did (B). (C–F) AUC of non-responder and responders after the first injection (C LH, and E FSH) and after the second injection (D LH, and F FSH) showed no difference. Data are expressed as median and interquartile range. NR non-responders, R responders, AUC area under the curve, C6 synthetic kisspeptin analogue, P4 progesterone.

Overall comparison of LH and FSH levels between responders and non-responders

When AUC data of LH and FSH of the second injection at different time intervals were pooled following the non-responder vs responder criteria, no difference was observed between groups (P = 0.27, U = 169 and P = 0.84, U = 207 for LH and FSH, respectively) (Fig. 7A and B).

Discussion

From progesterone to the kisspeptin system

We showed that a double injection of the Kp analogue C6 induces ovulation and a luteal phase in the ewe during the non-breeding season in the absence of progestogen and eCG treatments. Association of progestogen and eCG represents the benchmark in ovine reproduction management. Since many years, however, the use of these two hormones has been questioned. The potential neuroendocrine disruptor effect of progestogen and animal welfare issues related to eCG production are major issues (Decourt et al., 2019). To overcome these problems, we investigated the possibility of triggering ovulation by stimulating the Kp system with a synthetic compound: C6. We have previously shown that after a single injection of a formulation containing C6, the ratio of ovulation induction was lower than that of the benchmark treatment (Salzano et al 2022). A possible explanation would be insufficient follicle maturation. Follicle sensitivity to LH stimulation is under FSH control (Bartlewski et al., 1998) and injection of Kp or C6 in small ruminants produces an increase of FSH (Caraty et al., 2007; Decourt et al., 2019). We reasoned that an initial C6 treatment triggering FSH release would promote follicle growth to an LH–sensitive status. Hence, a second stimulation increasing LH should be able to trigger ovulation, induce follicle luteinisation and promote progesterone production. Nevertheless, there is a lack of information on the amount of FSH and the time required to induce follicular growth and sensitivity to LH for the Ile de France breed. Hence, we tested two combinations of doses (2.5+15 and 15+15 nmol of C6) and 3 time intervals (24, 36 and 48 h) to ascertain which will be more effective.

When combining 2.5 and 15 nmol doses, ovulation and a luteal phase were obtained only when 48 h had elapsed between the first and second injection. In addition, this was limited to 25% of treated ewes. Conversely, the combination of 15 and 15 nmol dose triggered some ovulation regardless of the interval of time between injections. Noteworthy, the 48-h interval resulted in a better percentage of ovulating animals (62.5%) compared to the other two intervals (37.5% and 25% for the 24 and 36-h intervals, respectively). The best percentage was also slightly superior to that previously obtained with a formulation containing C6 (Salzano et al., 2022).

Looking for a predictive LH and FSH signature of ovulation induction

To assess if a specific signature of plasma FSH and LH concentration could be established to discriminate between non-responders and responders, we followed the kinetics of the two hormones up to 24 h after the second injection. Our hypothesis was that the first injection would prime the reproductive axis and that a difference between responder and non-responder might

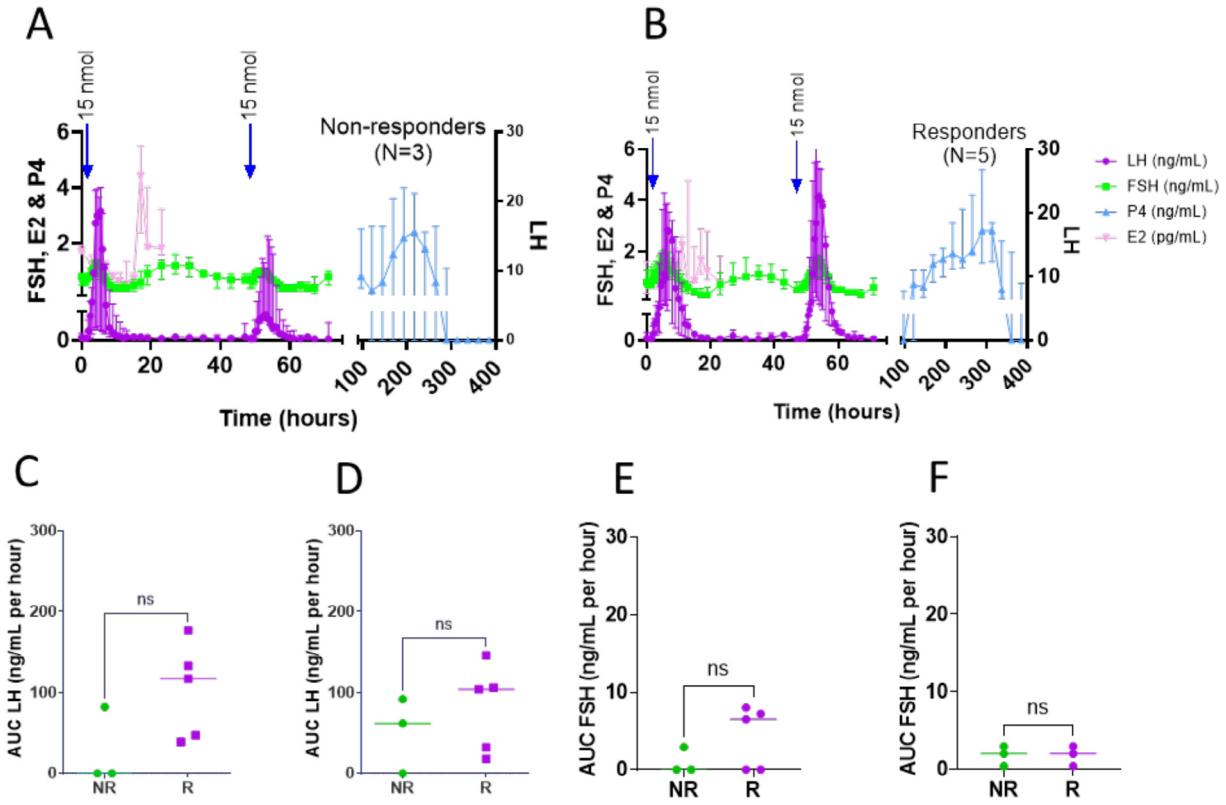


Fig. 6. Hormonal profiles induced by two injections of 15 nmol of C6 48 h apart. (A–B) LH, FSH, estradiol, and progesterone plasma levels following two C6 injections (blue arrows) 48 h apart. Both injections induced an increase in LH and FSH plasma levels. After the first injection, an increase of estradiol of variable magnitude was observed in both responders and non-responders. Three ewes showed a progesterone increase not fitting the criteria for responder (A) (progesterone level should be above 0.5 ng/mL for a least 9 consecutive days and mean progesterone value of the consecutive days should be above 1 ng/mL) whereas 5 did (B). (C–F) AUC of non-responder and responders after the first injection (C LH, and E FSH) and after the second injection (D LH, and F FSH) showed no difference. Data are expressed as median and interquartile range. NR non-responders, R responders, ns not significant, AUC area under the curve, C6 synthetic kisspeptin analogue, E2 Estradiol, P4 progesterone.

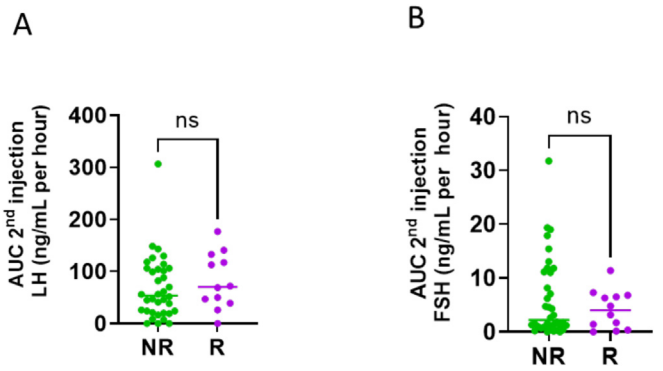


Fig. 7. Comparison of amount of LH and FSH released by responders and non-responders. (A–B) Regardless of the time interval between injections, the total amount of LH (A) and FSH (B) released by ewes after the second injection of 15 nmol of C6 was similar for responders (N = 12) and non-responders (N = 36). Data are expressed as median and interquartile range. NR non-responders, R responders, ns not significant, AUC area under the curve, C6 synthetic kisspeptin analogue.

be visible in response to the second injection. As expected, no difference was observed comparing data of the first injection. However, after the second injection, we also found no significant difference between non-responders and responders in the total amount released or the maximal amplitude of both LH and FSH. During an ovulatory cycle, the amount of LH leading to ovulation in ewes is highly variable between individuals. It is therefore plausible that this inter-individual difference could have masked a correlation between LH level and ovulation. Nevertheless, it was reported using a different experimental setting that the amplitude of LH surge was not related to ovulation induction and normal luteal function (McNatty et al., 1981). Despite this lack of correlation, ovulation and luteal phases were clearly a consequence of the treatment because none of the control ewes ovulated or had a significant change in their hormonal profile.

Considering the role of oestrogens

In ewes, starting a few hours after the beginning of a Kp perfusion, an increase of E2 triggering a "spontaneous" LH surge was observed (Sebert et al., 2010). From the data of this present study, we inferred that the time interval between treatments is likely the main factor influencing the capacity to induce a luteal phase. Hence, it seems unlikely that the first injection would trigger an increase in estradiol, leading to a "spontaneous" LH surge, inducing ovulation. To rule out this hypothesis, we checked E2 levels after the first C6 injection. Because injection after an interval of 48 h proved to be more effective in inducing a luteal phase, we analysed E2 levels only for this time interval. E2 concentrations were similar between responders and non-responders, making it impossible to discriminate between the two groups based on E2 plasma concentration. These results support the hypothesis that an increase of E2 in the first 24 h after the first injection is not involved in triggering ovulation and the luteal phase.

Effect of injection timing on LH and FSH response to the synthetic kisspeptin agonist C6

We investigated whether the first injection would influence, positively or negatively, the response to the second injection. As

expected, when injections were separated by 24 or 48 h, the first injection of C6 (2.5 nmol) triggered a small increase in LH compared to the second injection (15 nmol). At the 36-h interval, the trend was similar but not statistically significant. When injections of the same dose (15 nmol) were separated by 24 h, the second dose was less effective compared to the first one. Conversely, no difference was observed when injections were separated by 36 or 48 h. Previous studies in ewes using multiple Kp10 injections (1 each hour during 6 h) resulted in a similar increase of LH after each injection (Caraty et al., 2007). However, the effect of those injections on LH lasted less than an hour. Considering the longer duration of C6 effect, we could suppose that some form of desensitisation of the system would take place when injections were spaced of 24 h; such an effect would have disappeared at 36 and 48 h. Supporting this hypothesis, human studies showed that the capacity to stimulate LH and FSH release decreased rapidly when a regimen of twice-daily injections of Kp54, which has a longer half-life compared to Kp10 (d'Anglemont de Tassigny et al., 2017; Dhillo et al., 2005), was implemented. Conversely, no decrease in effectiveness was observed when injections were performed twice a week (Jayasena et al., 2010).

As expected, for the combination of 2.5 plus 15 nmol at 24 h, FSH increase was larger after the second injection, but no difference was observed at 36 and 48 h. The first C6 injection leads to a peak of FSH plasma concentration declining over about 10–12 h. This peak was followed by a second "spontaneous" FSH increase with a longer duration but smaller amplitude starting about 24 h postinjection. The second injection, when performed 24 h after the first, was indeed almost overlapping with the beginning of this "spontaneous" FSH increase. Conversely, at 36 h, the FSH was still relatively high and at 48 was declining. The fact that we observed a difference only at 24 h between the first (2.5 nmol) and the second injection (15 nmol) may be linked to this different initial level of FSH. A higher FSH level at 36 h may result in a reduced response to C6. Supporting this hypothesis, when injecting two doses of 15 nmol 36 h apart, the first dose triggers a larger FSH release compared to the second one. On the other hand, the reason for the lack of difference at 48 for the 2.5 plus 15 nmol combination of doses is not clear. Further investigation would be required to clarify this point.

Location of the action of the synthetic kisspeptin agonist C6

The use of repeated injections of GnRH, LH, FSH, and LH+FSH to induce ovulation in ewes was driven by the hypothesis that repeated injections would simulate the physiological episodic release of these hormones. For example, four GnRH injections at 90-min intervals followed by a fifth injection 5 h later produced an LH surge and ovulation. However, analysis of progesterone levels showed that luteal function was insufficient (Crighton et al., 1975). Similarly, LH, FSH or LH+FSH multiple injections during a 72–h period resulted in ovulation, but the occurrence of normal corpus luteum function was low (Wallace et al., 1986). Conversely, injection of GnRH every other hour for 40–80 days induced a return to cyclicity with normal luteal function (McNatty et al., 1982).

Considering that the Kp system is upstream of GnRH, and consequently of LH and FSH release, it is surprising that C6 produces a better outcome in terms of luteal function than multiple injections of GnRH, LH and/or FSH. It is likely that other factors play a role in the induction of luteal function by C6 treatment. In rats, an increase of Kiss1 expression was observed in the ovary during the preovulatory phase (Castellano et al., 2006), suggesting a possible local role of the Kp system in the preovulatory and ovulatory phase of the oestrous cycle. This increased expression was supposed to be driven by the LH surge (Castellano et al., 2006). Preovulatory surge of LH was also proposed to increase the production of Kp and the neurotrophin brain–derived neurotrophic factor in the ovary. In the mouse ovary, a coordinated action between the Kp and the neurotrophin system was reported. In the absence of the Kp signalling mouse oocytes do not upregulate the neurotrophin receptor NTRK2. This receptor is involved in follicular organisation and oocyte development, essential for correct follicle survival (Dorfman et al., 2014). It is tempting to speculate that the effect of C6 would combine the stimulation of GnRH release, triggering an increase of LH favouring a C6 direct action in the ovary. The efficacy of C6 in inducing a luteal phase would hence depend on a precise follicle physiological condition ready to receive both a gonadotropin and Kp stimulus. Nevertheless, we need to remain cautious as this hypothesis is based on data obtained from rodents, while data from the ewe would be essential to support it.

Conclusions

We showed that two injections of 15 nmol of C6 with a 48-h interval trigger an ovulation and a luteal phase in more than 60% of treated ewes. The LH, FSH and E2 profiles triggered by treatment are not predictive of animals that would respond with a luteal phase. We suggest that two factors might be important for a successful treatment with C6: (i) the status of progression in the follicular wave at the moment of the first injection and (ii) the physiological changes induced in the follicle by the first injection that would dictate the exact timing for a successful second injection. Additional work on the effect of C6 on ovary physiology would help test this hypothesis. Finally, it is clear that a progestogen priming is not mandatory to reactivate the reproductive axe during seasonal anoestrus. Stimulation of the Kp system by C6 is sufficient, even though not all ewes respond to the treatment with an appropriate luteal phase. Additional research is warranted to establish the possibility of using only C6 to match the performance of the current benchmark method.

Ethics approval

Experiments were carried out according to national and international regulations (Directive 2010/63/UE). All procedures were approved by the local Animal Ethics Committee (Comité d'Ethique en Expérimentation Animale Val de Loire) and authorised by the French Minister "de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche" (authorisation number 01873.03 and APAFIS#10958-2017062714349167v7).

Data and model availability statement

None of the data were deposited in an official repository. Data are available to the public institutions 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.

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Declaration of interest

M. Beltramo and V. Aucagne are inventors in a patent held by INRAE and protecting C6.

Acknowledgements

The Authors thanks the staff of INRAe UEPAO (Nouzilly; DOI: https://doi.org/10.15454/1.5573896321728955E12) for taking care of experimental ewes.

We thank Ross D. Ballantine for English editing.

Financial support statement

This work was supported by INRAe-Transfert : "Prematuration INRAe" grant held by MB.

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