Stimulation of LH secretion by 0.5 µg/kg cKP10 in various stages of the estrous cycle and anestrus in the Beagle bitch.

C. Buitelaar*
*Solis ID: 3258734, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.
Supervisor: Drs. C.H.J. Albers-Wolthers.

ABSTRACT

Background: Kisspeptins are recognized to be regulators within the hypothalamus-pituitary-gonadal axis and stimulators of GnRH and gonadotrophin secretion in mammals. In mice and sheep it is shown that mRNA expression of the *Kiss1* gene and *Kiss1R* gene, as well as plasma LH concentrations varies between cycle phases. Little is known about sensitivity to kisspeptin between different cycle phases in the dog. Therefore the aim of the study is to find out whether there is a difference in sensitivity to kisspeptin throughout the different stages of the canine estrous cycle and anestrus.
Methods: Beagle bitches were treated intravenously with cKP10 (0,5 µg/kg) during anestrus, follicular phase and luteal phase (PIP and PDP). Plasma LH concentrations are measured.
Results: In each experimental phase, plasma LH concentrations increased significantly after cKP10 administration. LH response peaks were higher during anestrus and PDP compared to follicular phase and PIP.
Conclusion: LH responses show that sensitivity to kisspeptin differs during the estrous cycle and anestrus of the Beagle bitch. LH response is high during anestrus and PDP compared to follicular phase and PIP. More research should be done to confirm our findings and the meaning of high plasma LH response during PDP should be further investigated.

INTRODUCTION

All over the world there is an enormous population of stray animals. In the United States alone, 4-5 million animals are euthanized in animal shelters each year due to a lack of homes (Kutzler, Wood 2006). Despite of the work of many charity organizations, which raise awareness for animal welfare and organize castration projects in countries all over the world (Kutzler, Wood 2006, Brown 2012, Anonymous 2011), no solution has been found for these growing problems. These problems include euthanizing millions of homeless animals each year and the reservoir of vectors of transmissible diseases to man and domestic animals formed by these stray cats and dogs (Kutzler, Wood 2006)
Until this day, one tries to solve the problems with the stray animal population with local trap and neuter programs. However this surgical method is time-consuming, costs a lot of money and is often done in an animal-unfriendly way (Kutzler, Wood 2006). Other solutions must be found. This solution should care for a life long lasting reproductive intervention in an animal-friendly way, which can be used on a wide scale. Chemical castration or long lasting estrus prevention could provide the means to reach this solution. Nowadays there are a few pharmaceuticals available for the use of estrus prevention or chemical castration. Progestagens, androgens and GnRH agonists are used for estrus prevention and these pharmaceuticals have a lot of side effects. Progestagens may induce dependent on the type of progestagen and the dose pyometra, mammary hyperplasia/neoplasia and diabetes mellitus in female dogs and cats. In male dogs and cats, progestagens only work in high dosages (Kutzler, Wood 2006). The use of androgens isn't registered in most countries and causes many side effects such as increase of libido, higher incidence of priapism and growth of prostatic tumors in the male dog and clitoral hypertrophy and vaginitis

in female dogs (Romagnoli, S. 2009). GnRH agonists have not only the disadvantage, that an estrus is induced within 1 to 4 weeks following the start of treatment in female dogs and cats, but also side effects as persisting estrus and endometritis. Treatment with GnRH agonist can only be initiated within a period of 60 days following an ovulatory estrus of within 7 days following parturition. GnRH agonists can also be used in male dogs, but there is a problem with differences in the duration of action (Kutzler, Wood 2006). Immunocontraception and intratesticular injections with $CaCl_2$ or Zinc Gluconate solution are used for chemical castration. Targets for the antibodies with immunocontraception are GnRH, LH and its receptors in both male and female dogs and cats, sperm antigens in male dogs and cats and oocyte zona pellucida in the female dog. More research to this methods should be done, because no reliable vaccines for dogs and cats are available (Kutzler, Wood 2006, Jana, Samanta 2007, Oliveira,E.C. 2012).

In conclusion, there is a need for a single dose, safe and effective method for contraception and estrus prevention in dogs and cats. The pharmaceuticals, which are available don't reach these goals. Kisspeptin and its antagonists could therefore provide a new target and an alternative method for non-surgical contraception in dogs and cats.

The discovery of kisspeptin was the result of research in humans with idiopathic hypogonatropic hypogonadism (IHH). In these patients with absence of pubertal development, due to low levels of sex steroids and gonadotropins, mutations in the Kiss1 receptor (Kiss1R) were noticed (de Roux et al. 2003). Similar loss of function mutations in the *kiss1R* gene are found in mice. Due to these mutations male and female mice don't undergo pubertal development and have low LH and FSH levels in their bloodstream. Mice with mutations in the *kiss1* gene show the same clinical signs (d'Anglemont de Tassigny et al. 2007, Chan et al. 2009) .

The reproductive function of mammals is regulated by hormonal messengers and feedback loops within the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotropin releasing hormone (GnRH) is secreted pulsatile by the hypothalamus and delivered to the anterior pituitary. Here, GnRH stimulates the gonadotrophic cells to produce luteinizing hormone (LH) and follicle stimulating hormone (FSH). These gonadotropins are transported into the circulation to the gonads, where testosterone and estrogens are produced. These sex steroids are required for spermatogenesis and oogenesis (Colledge 2008, de Gier et al. 2006).

Kisspeptins are small neuropeptides which are encoded by the *Kiss1* gene. The protein encoded by the *Kiss1* gene is a 145 amino-acid protein which is cleaved into the biologically active 54 amino-acid protein kisspeptin 54 (KP54), which is also known as metastatin. This KP54 is further degraded to KP14, KP13 and KP10. KP10 is the smallest biologically active kisspeptin. The amino acid order of all kisspeptins has the same carboxy terminal ending, which is responsible for the binding to the receptor. This receptor is Kiss1R (d'Anglemont de Tassigny et al. 2007, Castano et al. 2009, Gottsch, Clifton & Steiner 2009) .

The binding of the kisspeptin to the Kiss1R on the GnRH neuron is needed for stimulation of GnRH release and the subsequent release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the pituitary (Gottsch, Clifton & Steiner 2009, d'Anglemont de Tassigny et al. 2007) .

Central and peripheral administration of kisspeptin stimulated gonadotropin release in human (Dhillo et al. 2007, George et al. 2011), goats (Saito et al. 2012, Hashizume et al. 2010), calves (Ezzat Ahmed et al. 2009), monkeys (d'Anglemont de Tassigny et al. 2007) and rodents (Mikkelsen et al. 2009, Irwig et al. 2004).These results show that kisspeptin and the Kiss1R are essential for the reproductive function.

Kisspeptin and Kiss1R appear to be also very important in the feedback mechanisms of the HPG-axis. Until the discovery of kisspeptin it was unknown how the feedback mechanism of the HPG-axis worked, because the GnRH neurons don't possess the receptor for estrogens and testosterone. The majority (up to 90%) of the GnRH neurons in mammals does possess Kiss1R (Colledge 2008). The kisspeptin neurons do possess the estrogen, androgen and progesterone receptor (d'Anglemont de Tassigny et al. 2007). Kisspeptin is proven to be the missing link in the feedback mechanism of the HPG-axis (Garcia-Galiano, Pinilla & Tena-Sempere 2012, d'Anglemont de Tassigny et al. 2007) .

In adult castrated male mice the *kiss1* gene mRNA expression in the ARC was significantly higher compared

with intact male mice (Irwig et al. 2004, Navarro et al. 2004). In ovariectomized ewes, the number of *Kiss1* gene mRNA expressing cells increased significantly. The level of *Kiss1* gene mRNA expression on each cell increased also significantly. This increase in the number of *Kiss1* gene mRNA expressing cells, as the increase of the level of *Kiss1* gene mRNA expression could be lowered by estrogen or progestin treatment (Smith et al. 2007). These results show kisspeptin is very important for the HPG-axis feedback mechanism and therefore an interesting target for contraception and estrus prevention in dogs and cats.

The role of kisspeptin in the dog is unknown. However, preliminary data show kisspeptin administration increases plasma luteinizing hormone (LH) concentrations in the dog. Recently the dosage of 0.5 µg/kg cKP10 has been found by Karin Albers-Wolthers. This dosage was the result of a dose-finding study where 0, 0.1, 0,5, 1 and 10 µg/kg cKP10 where tested. The dosage of 0.5 µg/kg cKP10 was the lowest dose, which gave a significant raise in plasma LH concentrations.
It's important to know if the cKP10 has the same effect throughout the whole cycle of the bitch. It is known that the kiss*1* gene and *kiss1R* gene mRNA expression peaked at the diestrus in adult intact female mice and the lowest levels of *kiss*1 gene mRNA expression were at estrus (Navarro et al. 2004). The research of Rometo (2007) confirms that the expression of the *Kiss1* gene increased in the hypothalamic infundibular nucleus in post-menopausal woman. The same results were seen in ovariectomized monkeys (Rometo et al. 2007). It is important to know the effect of kisspeptin in the various stages of the estrus cycle and anestrus in the bitch. We want to create an antagonist which works a long time and should work in any stage of the estrous cycle and anestrus. So we must gather as much information as possible about the interaction between kisspeptin and Kiss1R throughout the different stages of the estrous cycle and anestrus of the bitch.

The aim of this study was to determine whether there is a difference in sensitivity to kisspeptin throughout the different stages of the canine estrous cycle and anestrus.

MATERIALS AND METHODS

Peptides: cKP10 (YNWNVFGLRY) was synthetized by the American Peptide Company (Sunnyvale USA), dissolved in sterile saline solution and stored at -20°C.

Animals: In total 6 healthy beagle bitches where used in these experiments. The dogs were born and raised at the University of Utrecht, Department of Clinical Science of Companion Animals and they were used to laboratory environment and procedures, such as the collection of blood. They were housed in pairs in indoor-outdoor kennels and fed a commercial dog food once daily. Water was provided ad libitum.

Cycle stage determination: All dogs were examined three times weekly to check for the presence of serosanguineous vulvar discharge and vulvar swelling. When serosanguineous discharge is noticed, this moment is considered to be the onset of proestrus. Plasma progesterone concentration was measured thrice weekly from the start of proestrus until the day it reached values above 12,72 nmol/L and ovulation was assumed to occur. Ovulation is followed by the luteal phase which can be divided into two phases: the pituitary independent phase (PIP) and the pituitary dependent phase (PDP) in the non-pregnant bitch (Okkens et al. 1990, Concannon 2009). Anestrus was defined as the period when plasma progesterone concentration initially decreased below 3 nmol/L (de Gier et al. 2006).

Experimental design: The experimental design was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands.
A cKp10 stimulation test was performed once in the early follicular phase, once in the PIP (around 12 days after ovulation), once in the PDP (around 42 days after ovulation) and once in anestrus (around 100 days after ovulation).
During the experiment a single injection of cKP10 (0.5 µg/kg) was administered intravenously in the vena

cephalica at T=0. Blood was collected from the jugular vein and placed directly in heparinized tubes at T=-40, -30, -20, -10, 0, 10, 20, 30, 40, 50, 60 min after cKP10 administration. Plasma samples were obtained after centrifugation for 12 minutes, x 3500 G, at 5°C and stored at -20°C. Plasma LH concentrations were determined from each sample.
During the experiments, the dogs were closely monitored for side-effects: pulse rate, perfusion parameters (mucous membrane color and capillary refill time) and respiratory rate were monitored.

Hormone assay: Plasma progesterone concentrations were measured using a ^{125}I-radioimmunoassay (RIA) validated for the dog (Okkens,A.C. 2001). The intra-assay and interassay coefficients of variation (CVs) are 6% and 10.8%, respectively. The lower limit of quantitation is 0.15 nmol/L. Plasma LH concentrations were measured with a heterologous RIA as described previously (Nett et al. 1975). The intra-assay and interassay CVs for values above 0.5 µg/L are 2.3% and 10.5%, respectively, and the limit of quantitation is 0.3 µg/L.

Data analysis: An ANOVA for repeated measures was used to compare basal plasma LH levels with peak plasma LH levels within each examined group, peak concentrations between the examined groups and AUC between the examined groups. The data that were not normally distributed were log-transformed. The data were normally distributed after log-transformation. When significant differences were noticed, a Bonferroni test was used. AUC data were derived from plasma LH response curves in each of the examined groups. The basal plasma LH level was subtracted from the AUC with the assumption that basal plasma LH levels were the same in all the experiments. Results are presented as mean or mean ± SEM. The level of significance was $p < 0,05$.

RESULTS

Intravenous administration of 0.5 µg/kg cKP10 resulted in a raise in plasma LH concentrations in all animals used in this experiment (fig. 1). The means of the plasma LH concentrations in all the examined phases increased significantly when T=0 and T=10 are compared ($p < 0,05$). The raise in plasma LH concentration was highest during anestrus and the variance between plasma LH concentrations was high 10 minutes after cKP10 administration and at T=-20 (fig. 1A). The raise of plasma LH concentration at T=10 during anestrus seemed significantly higher compared to T=10 in the follicular phase and the PIP, however when Bonferroni's test was performed significance was lost. Plasma LH concentrations in PDP are significantly higher compared to the follicular phase ($p < 0,05$). Plasma LH concentrations in PDP were also higher compared to PIP, but this difference was borderline ($p = 0.08$). In all experiments the plasma LH concentrations return to basal level at T=60, except during the PIP (fig. 1C). During the PIP the variance is high at T=-40 and at T=50 and T=60. AUC analysis confirmed the LH response is greater during anestrus and PDP compared to the follicular phase and the PIP, however when Bonferroni's test was performed significance was lost (fig. 1F). There is no significant difference in the AUC between anestrus and PDP and between follicular phase and PIP.

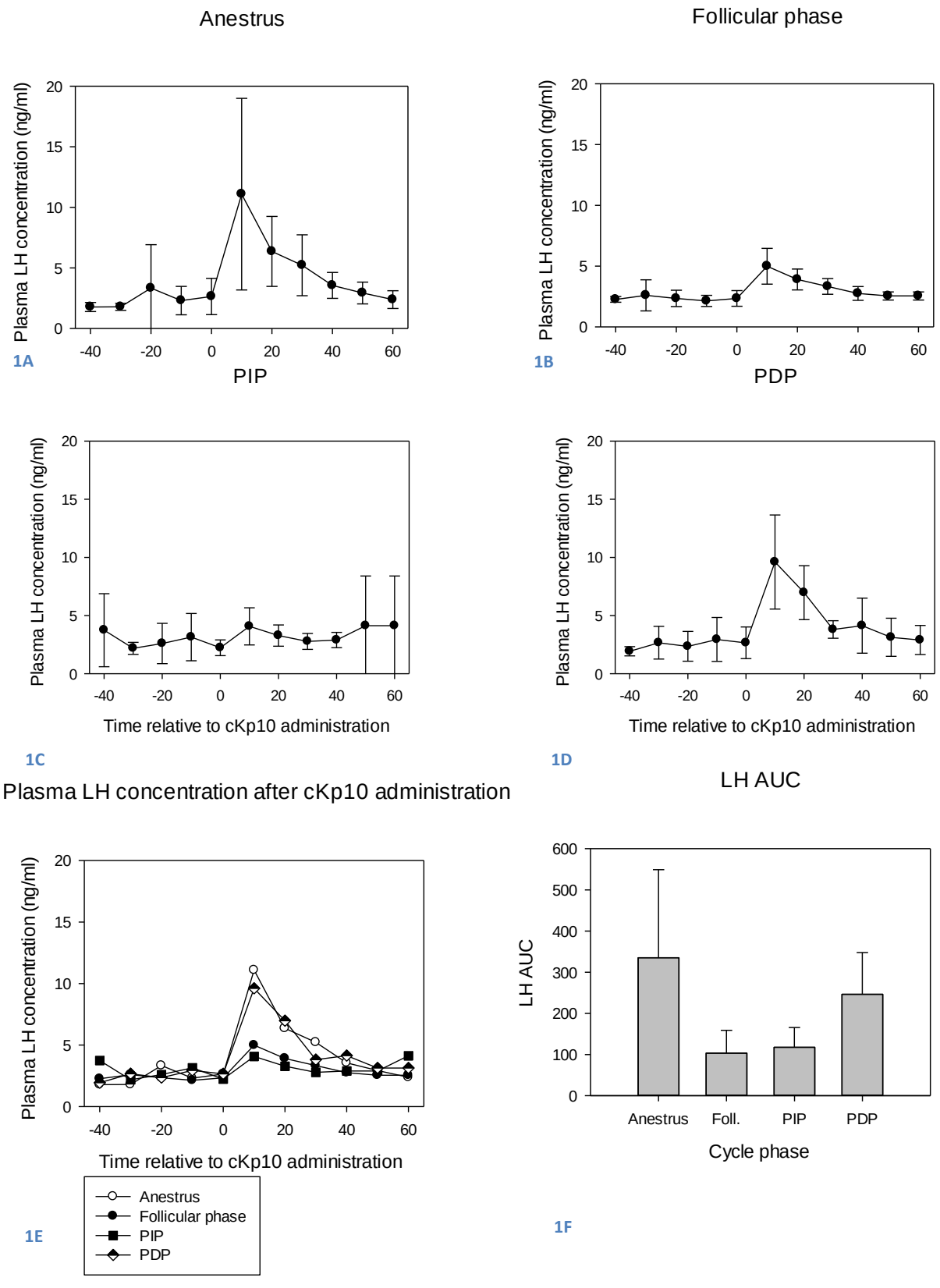

Fig.1. Treatments with intravenous cKP10 (0,5 µg/kg) were given at T=0. Plasma LH concentrations were measured during anestrus (A), follicular phase (B), PIP (C) and PDP (D). Anova for repeated measures showed a significant difference between LH response during PDP and follicular phase. AUC data did not show significant differences. AUC was calculated form LH response curves (-40 to 60 min).

DISCUSSION

The purpose of our study was to find out whether there is a difference in sensitivity to kisspeptin stimulation throughout the different stages of the canine estrous cycle and anestrus. The results show that there was a significant increase in plasma LH concentration after cKP10 administration in all stages of the estrous cycle and anestrus of the bitch.
It is shown that plasma LH responses in the dog are significantly higher in late anestrus (day 160 to 202 after ovulation) compared to early anestrus (day 90 – 132 after ovulation). This suggests that GnRH sensitivity increases during anestrus (van Haaften et al. 1994). Sensitivity to GnRH varies during anestrus. KP10 stimulates LH secretion, via the release of GnRH and therefore it would also be likely for KP10 to show variation in sensitivity.
Our results show that the raise in plasma LH concentration was higher in anestrus and PDP in comparison to early follicular phase and PIP. However when Bonferroni's test was performed, significance was lost. Only the plasma LH concentrations during PDP were significantly higher compared with the follicular phase. Plasma LH concentrations during PDP were higher compared to the PIP, however this significance was borderline (p =0,08). This is probably due to the great variance found in the data and the low number of participating animals in this experiment. Repeating this experiment with a greater number of participating animals could reveal that the trend we see in our data might be significant.
There is a high variance in LH response between individual animals within each examined group, especially in anestrus. However every individual dog showed an increase in plasma LH concentration after cKP10 administration. Some of the variance in basal plasma LH concentrations can also be explained by physiologic pulsatile secretion of LH by the pituitary gland. In the bitch LH is secreted pulsatile during all phases of the estrous cycle and anestrus by the pituitary gland.
The follicular phase has a relatively high basal plasma LH concentrations and the secretory pattern is characterized by frequent increases of short duration. During the second part of the luteal phase there is an increased frequency of endogenous LH pulses (Kooistra,H.S. 1999). These LH pulses can occur at any moment and could therefore be responsible for the high variance during the examined phases in our study. Also the high variance during PIP at T=-40, T=50 and T=60 could be explained by the endogenous secretion pattern of LH by the pituitary.
The AUC data confirm the trend in plasma LH concentrations at T=10 during anestrus and PDP. These values are higher compared to T=10 of the follicular phase and the PIP. The AUC data also show that the plasma LH concentrations during the whole experiment were higher during anestrus and PDP in comparison to the follicular phase and PIP.
Our findings suggest that there is a difference in sensitivity to kisspeptin throughout the different stages of the canine estrous cycle and anestrus, which is in agreement with other studies. Navarro et all (2004), showed that mRNA expression of the *kiss1* gene was the lowest during estrus in female mice. They also showed that ovariectomized female mice have a high mRNA expression of the *kiss1* gene and *kiss1R* gene and this effect could be voided with the administration of estradiol. Plasma LH concentrations were also high in ovariectomized female mice and this effect is also voided with the administration of estradiol (Navarro et al. 2004).
This is consistent with our findings that plasma LH concentration after cKP10 administration was higher in anestrus in comparison to follicular phase and PIP. Lower concentrations of estrogen and progesterone during anestrus contribute to a low level of negative feedback in the HPG-axis. Kisspeptin neurons could therefore be more sensitive to the intravenously administered cKP10. This could be in accordance with the higher expression of the *kiss1* gene and *kiss1R* gene in the ovariectomized female mice (Navarro et al. 2004). High expression of *Kiss1* gene mRNA is also found in post-menopausal women, ovariectomized female monkeys (Rometo et al. 2007) and in ovariectomized ewes (Smith et al. 2007). These differences in expression of the *Kiss1* gene and *Kiss1R* gene between the different phases could be responsible for the higher sensitivity to kisspeptin during anestrus. Smith et all (2009) confirmed our finding of an increased

plasma LH concentration during anestrus. They demonstrated in ewes, that the plasma LH concentration was significantly higher in anestrus compared to other stages of the reproductive cycle (Smith,J.T. 2009). Our finding that the plasma LH concentration in the PDP, besides anestrus was also higher compared to the follicular phase and PIP differs from other studies. However, there is one study which stated that pulsitility of LH secretion increased and peaks of higher amplitude were noticed during the second part of the luteal phase in the non-pregnant Beagle bitch (Onclin,K. 2002).
Navarro et all (2004) did find a peak in mRNA expression of the *kiss1* gene and the *kiss1R* gene in the early luteal phase in mice (Navarro,V.M. 2004). This is inconsistent with our finding of lower plasma LH concentrations during PIP and a peak in plasma LH concentration during PDP. This inconsistency could be due to the fact that in our study plasma LH concentrations are measured, instead of mRNA expression levels. It could also be due to interspecies variations in plasma LH concentrations.

We found a difference in LH response between the two stages of the luteal phase. The LH response was higher in the PDP compared to the PIP. Prolactin is an important luteotrophic factor during the PDP in the bitch (Kowalewski,M.P. 2012). It is proven that kisspeptin 10 stimulates the secretion of prolactin in bovine anterior pituitary cells in vitro (Ezzat, A.A. 2010, Kadokawa,H. 2008). Therefore it could be possible that sensitivity to kisspeptin is increased during PDP, needed for an increase in luteotrophic prolactin to maintain the corpus luteum. The increased sensitivity to kisspeptin during PDP is also responsible for the high plasma LH concentrations after cKp10 administration during our experiment. However, kisspeptin failed to influence circulating prolactin levels in Rhesus monkeys (Ramaswamy,S. 2009).
Another cause for the high LH response during PDP in comparison to PIP, could be that kisspeptin is important in the process of luteal regression. Luteolysis in the non-pregnant bitch is very poorly understood, in contradiction to the pregnant bitch, where PGF2a is the main luteolythic factor (Kowalewski,M.P. 2012, Hoffmann,B. 2004). It is suggested that modulations in the expression of COX 1 and COX 2, together with the paracrine action of progesterone are responsible for the luteal regression (Hoffmann,B. 2004). However blockage of luteotrophic prolactin by a dopamine agonist has also shown to result in irreversible luteolysis (Onclin,K. 1993). If kisspeptin could influence prolactin levels during PDP, it could be responsible for luteal regression at the end of the PDP.
Szawka et all (2010) stated that kisspeptin neurons within the arcuate nucleus of the hypothalamus of adult male and female mice played an important role in the regulation of dopamine and thereby prolactin secretion by the hypothalamus. They found that intracerebroventricular kisspeptin administration increased prolactin release. This KP10 induced prolactin release was associated with a decreased dopaminergic activity in the median eminence. Kisspeptin fibers were also observed in close apposition to dopaminergic fibers of the ARC (Szawka,R.E. 2010). In this research kisspeptin administration resulted also in prolactin release. This suggests that kisspeptin is able to alter the prolactin secretion by the hypothalamus. This could mean that kisspeptin is responsible for luteal regression at the end of PDP, by decreasing circulating prolactin concentrations. Much more research should be done to investigate this possibility.
Now that we know sensitivity to kisspeptin differs during the various stages of the estrus cycle and anestrus in the dog, more research is needed to investigate the role of kisspeptin within the canine estrous cycle. This experiment must be repeated in the future, with a higher number of participating animals to make sure the data we found could be significant. Further research is needed to investigate the meaning of high plasma LH concentrations in the PDP after cKP10 administration and the consequences for luteal regression.
More research should clarify, whether kisspeptin or its antagonists could be an alternative for non-surgical contraception in the dog. Studies with kisspeptin antagonists must be performed to find out whether antagonistic effect could be provoked and dose studies must be performed to establish the optimal dose. More studies with kisspeptin antagonist must be done in different stages of the canine estrous cycle and anestrus.

In conclusion we discovered that there is a difference in sensitivity to kisspeptin during the various stages of the estrous cycle and anestrus in the dog. Results show that intravenous cKP10 administration causes a raise in plasma LH concentration during anestrus and PDP compared to the follicular phase and PIP in the Beagle bitch. Plasma LH concentrations during the PDP are significantly higher compared to the follicular phase. This could be due to differences in mRNA expression of Kiss1 and Kiss1R.

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