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Abstract

Leptin regulates body weight and several physiological processes including reproduction. We evaluated the circulating levels of leptin in pregnant and non-pregnant bitches as well as their correlation with body weight, food intake, and number of fetuses. Nineteen healthy German Shepherd bitches were used and divided in two groups (pregnant n=12 and non-pregnant n=7). Blood samples were collected every 15 days starting from ovulation (Day 0) throughout pregnancy (pregnant group, P) or throughout luteal phase (non-pregnant group, NP) In pregnant bitches, leptin concentrations increased from the day of ovulation (1.32±0.06 ng/ml) up to day 45 (1.51±0.06 ng/ml; $P<0.01$) and returned to baseline values from day 60 post-ovulation. In non-pregnant bitches, leptin concentrations remained constant throughout the whole observation period (estimated marginal mean±SE=1.33±0.38 ng/ml). Pairwise comparisons showed significant differences between P and NP at day 45 post-ovulation ($P<0.05$). Multivariable models indicated that, controlling for time and litter size, there was a positive relationship between leptin concentration and BW ($P<0.05$) although Pearson coefficients showed that the correlation between BW and leptin was only significant in NP animals at day 45 ($r=0.76$, $P<0.05$). The multivariable approach also suggested that, holding BW and time constant, leptin concentrations tend to increase as the number of puppies increased ($P=0.06$). Our study supports indirectly the contribution of the feto-placental unit to the circulating maternal leptin concentration.

Introduction

Leptin, a member of the family of cytokine, is a 167 amino acid polypeptide considered to play a crucial role in the regulation of energy homeostasis and body weight (Wauters et al., 2000). Encoded by the obese gene (OB), it is mainly synthesized by adipocytes (Wada et al., 2014). However, the presence of cognate receptors in several other organs including liver, pancreatic β cells, skeletal muscle, T lymphocytes, and epithelial cells, suggests that leptin is also involved in the regulation of a wide range of functions including glucose metabolism (Wauters et al., 2000) and several other physiological processes from inflammation to angiogenesis, hematopoiesis and immune function (Fantuzzi et Faggioni, 2000; Boulomie et al., 1998)

Finally, the localization of leptin and/or leptin receptor in the fetus and placenta of several mammals including humans (González et al., 2000), sheep (Thomas et al., 2001), pigs (Smolinska et al., 2007), mice, and rats (Hoggard et al., 1997) suggests that leptin may have a role in the control of energy homeostasis during pregnancy. The profiles of leptin blood concentrations during pregnancy have been extensively described in numerous mammalian species such as humans, sheep (Thomas et al., 2001), cattle (Block et al., 2001), goats (Bonnet et al., 2005), pigs (Saleri et al., 2015), rats (Tomimatsu et al., 1997), and rabbits (Menchetti et al., 2015). During pregnancy, increased levels of leptin likely guarantees the mobilization of maternal fat reserves to cope with fetal growth, supporting the development of fetal neuronal networks and acting as a real growth factor for the embryo (Hassink et al., 1997; Lin, 1999; Denis et al., 2003; Haugel-de Mouzon et al., 2006; Desai et al., 2011).

More recently, Balogh et al. (2015) reported that leptin and its receptor were expressed in the uterus and placenta of pregnant dogs while Cardinali et al. (2017) reported an increase in leptin concentration during bitch's gestation. In this publication, the authors describe a profile of leptin similar to that already reported in humans (Hardie et al., 1997), rats (Tomimatsu et al., 1997) and rabbits (Menchetti et al., 2015) characterized by a gradual increase in serum levels during the first half of the pregnancy to reach peak concentrations halfway through gestation. A similar condition of pregnancy associated hyperleptinemia in women was attributed to the placental production of leptin (Lin, 1999; Hardie et al., 1997).

The aims of the present work were to compare, for the first time, the circulating levels of leptin in pregnant and non-pregnant bitches as well as their correlation with body weight, food intake, and number of fetuses.

Materials and methods

Bitches

The study was approved by the Ethical Committee of the University of Perugia (Prot. 2019-04R) and performed with owner consent in accordance with Italian laws and EU directives as a part of normal veterinary clinical practice.

For this study, we recruited 19 German Shepherd bitches ranging in age from 2 to 9 years (median 4.7 years) and weighing 29 ± 1 kg. The privately-owned dogs were followed by the Obstetric and Gynecology Service of the Veterinary Teaching Hospital of the University of Perugia. Based on clinical examinations and reproductive history, dogs were considered as healthy with normal inter-estrus intervals. All dogs had had at least one previous litter.

From the first appearance of vulvar serosanguineous discharges, indicating the onset of proestrus, vaginal smears were taken daily and examined using hematoxylin-eosin staining to identify the end of proestrus. Progesterone evaluation was used to calculate the estimated LH surge and ovulation. The day of ovulation (day 0) was calculated from the estimated LH peak as the first day when plasma progesterone was over 10 ng/ml (Brugger et al., 2011). Pregnancy was diagnosed 20 to 25 days after ovulation by conventional B-mode ultrasonography using a MyLab 30 Gold system (Esaote; Genoa, Italy) equipped with a 5.5-7.5 MHz-microconvex probe. Sonography was repeated every 15 days to monitor fetal health.

The bitches were allocated to two groups: pregnant group (P, n=12) and non-pregnant group (NP, n=7). The bitches in the P group were mated twice every 48 h from estimated day of ovulation whereas the bitches of the other group remained unmated.

All bitches were fed twice daily (at 8.00 a.m. and 8.00 p.m.) using a commercial diet (Sanypet S.p.a., Bagnoli Di Sopra (PD) Italy) according to physiological state. In particular for pregnant bitches until day 40 and non-pregnant bitches, an average energy demand of 132 kcal/kg

$BW^{0.75}$ was estimated. The diet used in this study was complete and balanced REPRODUCTION FEMALE dry food (Sanypet S.p.a., Bagnoli Di Sopra (PD) Italy). REPRODUCTION FEMALE is characterized by a omega-6:omega-3 ratio of 4:1 and the presence of active substances Maca (Lepidium meyenii), beta-carotene, folic acid, and L-carnitine that were contained in heart shaped tablets

From day 40 of gestation and up to parturition, only the pregnant bitches received a supplementation of 26 kcal/kg BW. These amounts were sufficient to meet the metabolic energy needs based on F.E.D.I.A.F. recommendations (Nutritional guidelines for complete and complementary pet food for cats and dogs, August 2011). In particular the diet used, for pregnant bitches from the day 40 until delivery, was PUPPY CONDRO ACTIVE which is complete and balanced dry food for puppies from 2 to 12 months that is characterized by a omega-6:omega-3 ratio of 1:1 and active substances Boswellia serrata, Harpagophytum procumbens, Chondroitin sulphate (from shark cartilage) and vegetal glucosamine.

Food intake was assessed daily by weighing food before and after feedings by owners. No other source of food was available for the animals. Water was provided ad libitum. The body weights were recorded every 15 days at 7.00 a.m. before food administration. and blood sample collection at the Teaching Hospital of the University.

Blood sampling

Blood samples (2 ml) were collected from the cephalic vein daily from late cytological proestrus to ovulation and then every 15 days to day 75. Blood samples were drawn without anticoagulant, centrifuged at 3000 x g for 15 min, and sera stored at -20C° until assayed for progesterone and leptin.

Hormone assays

Serum progesterone concentrations were evaluated daily by ELFA kits (MiniVidas bio-Merieux, Florence, Italy) validated for canine (Brugger et al., 2011;).

Serum leptin concentrations were measured by double antibody RIA using the multi-species leptin kit (MO, USA EMD Millipore Corporation, Billerica, MA). The limit of sensitivity was 0.8 ng/ml and intra- and inter-assay coefficients of variation were 3.4 and 8.7%, respectively.

Statistical analysis

Diagnostic graphics were used to check assumptions. Due to lack of normality, logarithmic transformations were used for both leptin and BW values. Data were analysed by Linear Mixed models. In these models, "Time" (days post ovulation) was included as a repeated measure with a scaled identity covariance structure while the bitch was included as a subject factor. We first analysed the influence of the physiological state on leptin concentrations with a model that evaluated the effects of "Time" (6 levels: fortnightly sampling from day 0 to 75 post-ovulation), "Physiological state" (2 levels: P and NP), and interaction "Time" x "Physiological state" (Barbato et al., 2017; Menchetti et al., 2018). Sidak corrections were used for pairwise comparisons. The same model was used to evaluate changes in BW. Results were expressed as estimated marginal means (mean responses for each factor, adjusted for any other variables in the model)± standard error (SE). When appropriate, estimated marginal means were back-transformed while raw data were presented in figures.

Then a multivariable model was built to evaluate if the leptin concentration was influenced by the litter size adjusting for BW and time. This model evaluated the main effects of BW, Time, and Litter size included as a continuous variable (number of puppies, from 0 to 11 puppies). We reported the regression coefficient for fixed effects (B) with standard error (SE) and P value.

The Pearson test (r) was used to evaluate the correlation between BW and leptin according to the physiological state (P and NP). Correlation was defined as high when absolute value of $r > 0.5$, medium when r ranged from 0.3 to 0.5, and low when $r < 0.3$ (Field, 2009). Statistical analyses were performed with SPSS Statistics version 23 (IBM, SPSS Inc., Chicago, IL, USA). We defined $P \leq 0.05$ as significant and P-values between 0.05 and 0.1 as a trend.

Results

All bitches in the pregnant group whelped healthy puppies naturally 61-65 days post ovulation (median=63 days) and no signs of embryonic resorption or fetal death were displayed during the observation period by ultrasound.

Body weight

All dogs ate their daily portion of food completely without significant changes in overall food intake overtime. In the pregnant group, BW increased progressively up to the end of pregnancy ($P<0.01$) while no significant changes were observed in the non-pregnant group. At day 60 post ovulation, BW tended to be higher in pregnant than non-pregnant bitches ($P=0.1$; Fig. 1).

Univariable analysis of leptin concentrations

Estimated marginal mean of leptin concentrations were higher in P than NP ($P=0.04$). In pregnant bitches, leptin concentrations increased from the day of ovulation (1.32±0.06 ng/ml) up to day 45 (1.51±0.06 ng/ml; $P<0.01$) and returned to baseline values from day 60 post-ovulation (1.38±0.06 ng/ml). Conversely, leptin concentrations remained constant throughout the observation period in non-pregnant bitches (estimated marginal mean± SE =1.33±0.38 ng/ml). Pairwise comparisons showed that there were significant differences between the P and NP at day 45 post-ovulation (mean difference±SE =1.24±0.26 ng/ml; $P=0.02$; Fig. 2).

Multivariable analysis of factors affecting leptin concentration: effect of body weight, time, and litter size

Multivariable model (Table 1) showed that, controlling for time and litter size, there was a positive relationship between leptin concentration and BW ($P<0.05$). Moreover, leptin was significantly higher at days 30 and 45 compared to the day of ovulation ($P<0.05$) even after adjusting for other independent variables. Finally, the model showed that, holding BW and time constant, leptin concentrations increased as the number of puppies increased (as trend, $P=0.06$).

Correlation analysis of body weight and leptin concentration

A positive correlation was found between BW and leptin in non-pregnant animals at day 45 (r=0.76, P=0.05) and in pregnant bitches at day 0 (r= 0.56 as trend, P=0.08; Table 2) but not later in pregnancy.

Discussion

Leptin is considered a pleiotropic hormone as it regulates many physiological processes including reproduction by regulating ovarian function, oocyte maturation, embryo development, implantation, placentation, and immune mechanism at the maternal interface (Pérez-Pérez et al 2018). Interestingly, human (Dötsch, et al., 1999) rat (Kawai et al., 1997), and murine (Hoggard et al., 1997) placenta were found to be a source of leptin. More recently, Balogh et al. (2015) reported the gene expression of leptin and leptin receptor in the placenta of dogs.

Major changes of circulating leptin concentrations have been observed during pregnancy in women (Hardie et al., 1997), rats (Chien et al., 1997), rabbits (Menchetti et al., 2015), and bitches (Cardinali et al., 2017). According to Linneman et al. (2000) and Hauguel-de Mouzon et al. (2006), the placenta rather than maternal adipose tissue was contributing significantly to the higher levels of leptin detected in human maternal circulation. In contrast, there are still some controversies on whether placental-derived leptin would affect the maternal circulating levels in pregnant rodents (Hoggard et al., 1997).

In our study, the concentrations of leptin levels in pregnant bitches were also higher than those observed in non-pregnant animals. Indeed, in non-pregnant animals, no changes in body weight and in food intake were observed while leptin concentration did not change and remained constant. In pregnant animals, leptin was observed to increase slowly proportionally with the increase in food intake although a clearly significant body weight gain was observed only at day 60.

However, if the increase in leptin can be related to the increase in food intake and body weight, the contribution of the feto-placental unit to the circulating maternal leptin concentration can also as suggested by Gong et al (1996), be proposed.

Indeed, if we indirectly evaluate the contribution of the placentas, regardless of the other factors (using a multivariable model approach adjusting for BW and Time), we identify an association between leptin and litter size. The multivariate analyses demonstrated that, when holding BW and Time constant, the leptin plasma concentrations tend to increase as the number of puppies increased. The multivariable model also indicated that there is a positive relationship between BW and leptin. To further investigate the relationship between BW and leptin, we conducted Pearson correlation tests stratifying in pregnant and non-pregnant animals for each observation day. These tests showed that the correlation between BW and leptin was significant only in non-pregnant animals at day 45. There was no complete agreement between the results of multivariate and bivariate analyses.. Although it could be explained by the two different statistical approaches, a type 1 or 2 error cannot be ruled out as we expected a strong consistency in the relationship between BW and leptin, at least in non-pregnant bitches.

Anyway, the weak or absent relationship between BW and leptin during pregnancy as well as the positive association between leptin concentration and litter size suggest that (i) the adipose tissue may not be the main source of leptin during pregnancy and (ii) a role could be played by the presence and the number of placentas. This model however did not allow us to identify which of the dietary intake or changed physiological conditions (pregnancy) is the primary factor influencing leptin concentrations. The fact that relatively high leptin concentrations during pregnancy did not correlate with food intake changes suggests that the leptin-induced negative feedback on food-intake and energy homeostasis is overcome by pregnancy.

The return of leptin to baseline values may be related to the combined effects of placenta and fetal removals at parturition and the negative energy balance typical during this postpartum period. It may coordinate the neuroendocrine adaptations responsible for energy partitioning between mother and the growing fetuses and/or reflects the increase of food consumption during lactation (Block et al., 2001; Menchetti et al., 2015).

Balogh et al (2015) reported the expression of leptin and leptin receptor in the fetal and maternal sides of the placenta over the course of pregnancy. Their presence indicates multiple paracrine/autocrine functions (Balogh et al 2015). Our study may also provide further data for an endocrine role of leptin during pregnancy Further studies are needed to assess the role of placental and fetal production of leptin and its influence on maternal plasma concentrations regulation of

BW and control of food intake as reported for other species (Hoggard et al., 1997; Linnemann et al., 2000; Hauguel-de Mouzon et al., 2006).

Declaration of interest

The Authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1. Multivariate model including leptin concentrations (ng/ml, after logarithmic transformation) as the dependent variable showing the regression coefficient for fixed effects (B), standard errors (SE), and P values.

Parameter	**B**	**SE**	**P value**
Body weight			
(kg)[1]	0.405	0.188	**0.033**
Day post-ovulation			
Day 15 vs day 0	0.033	0.060	0.585
Day 30 vs day 0	0.128	0.063	**0.044**
Day 45 vs day 0	0.144	0.061	**0.019**
Day 60 vs day 0	0.013	0.065	0.836
Day 75 vs day 0	0.001	0.061	0.986
Litter size			
n° of puppies[2]	0.008	0.004	**0.057**

[1] After logarithmic transformation.

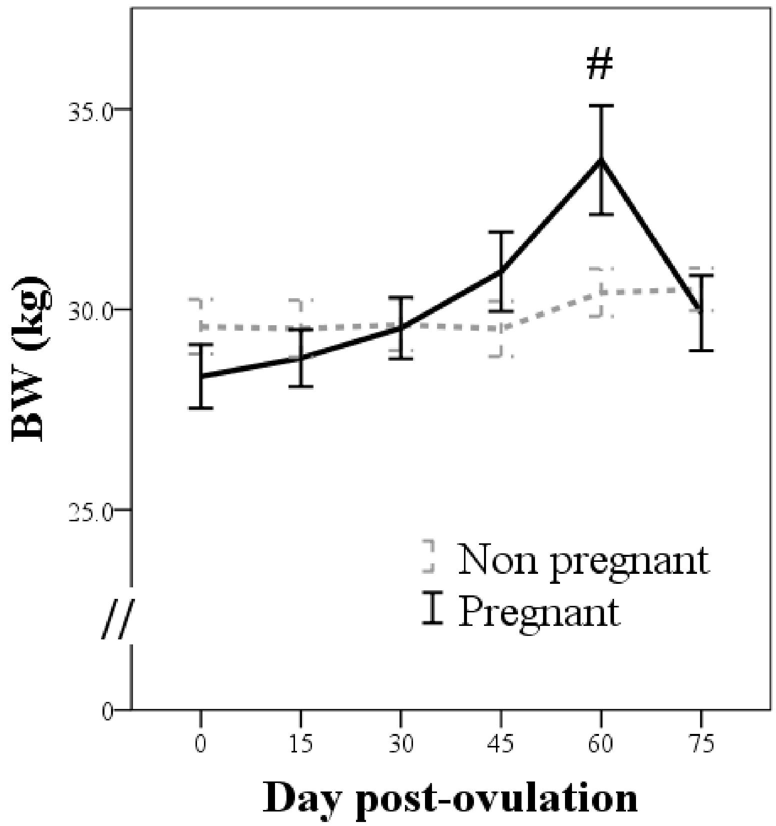
[2] ranging from 0 to 11 puppies, included as continuous variable.

P-value <0.1 are in bold.

Table 2. Correlations between body weight and leptin according to the physiological state and the day post-ovulation.

Physiological state	Day post-ovulation	Pearson correlation	P-value
No pregnant	**0**	0.338	0.459
	45	0.764	**0.045**
	75	0.438	0.326
Pregnant	**0**	0.556	**0.076**
	45	-0.093	0.813
	75	0.151	0.639

P-value <0.1 are in bold.



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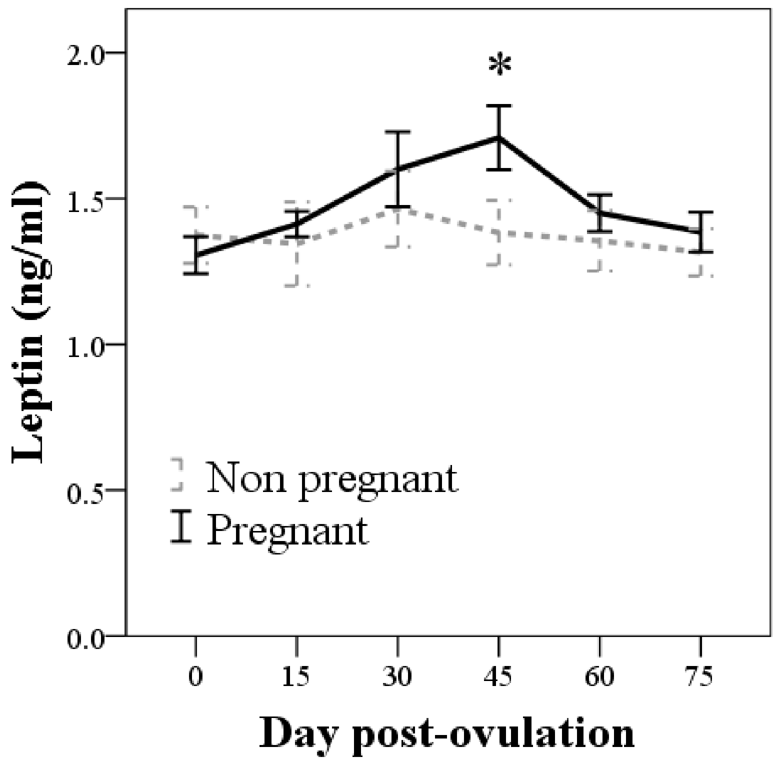

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