

## Insulin binding characteristics in canine muscle tissue: effects of the estrous cycle phases<sup>1</sup>

Álan G. Pöppl<sup>2,3\*</sup>, Sandra C. Valle<sup>3</sup>, Félix H.D. González<sup>4</sup>, Luiz C. Kucharski<sup>3</sup>  
and Roselis S.M. da Silva<sup>3</sup>

**ABSTRACT.** Pöppl A.G., Valle S.C., González F.H.D., Kucharski L.C. & Da Silva R.S.M. 2016. **Insulin binding characteristics in canine muscle tissue: effects of the estrous cycle phases.** *Pesquisa Veterinária Brasileira* 36(8):761-766. Setor de Clínica de Pequenos Animais, Hospital de Clínicas Veterinárias. Departamento de Medicina Animal, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. E-mail: [alan.poppl@ufrgs.br](mailto:alan.poppl@ufrgs.br)

Hormonal fluctuations during the different estrous cycle are a well-recognized cause of insulin resistance in bitches, and little is known about insulin receptor binding or post-binding defects associated with insulin resistance in dogs. To evaluate insulin binding characteristics in muscle tissue of bitches during the estrous cycle, 17 owned bitches were used in the study (six in anestrus, five in estrus, and six in diestrus). An intravenous glucose tolerance test (IVGTT) was performed in all patients by means of injection of 1mL/kg of a glucose 50% solution (500mg/kg), with blood sample collection for glucose determination at 0, 3, 5, 7, 15, 30, 45 and 60 minutes after glucose infusion. Muscle samples, taken after spaying surgery, were immediately frozen in liquid nitrogen and then stored at -80 °C until the membranes were prepared by sequential centrifugation after being homogenized. For binding studies, membranes were incubated in the presence of 20,000cpm of human <sup>125</sup>I-insulin and in increasing concentrations of unlabeled human regular insulin for cold saturation. The IVGTT showed no differences among bitches during the estrous cycle regarding baseline glycemia or glycemic response after glucose infusion. Two insulin binding sites - high-affinity and low-affinity ones - were detected by Scatchard analysis, and significant statistical differences were observed in the dissociation constant (*K*<sub>d1</sub>) and maximum binding capacity (*B*<sub>max1</sub>) of the high-affinity binding sites. The *K*<sub>d1</sub> for the anestrus group (6.54±2.77nM/mg of protein) was smaller (*P*<0.001) than for the estrus (28.54±6.94nM/mg of protein) and diestrus (15.56±3.88nM/mg of protein) groups. *B*<sub>max1</sub> in the estrus (0.83±0.42nM/mg of protein) and diestrus (1.24±0.24nM/mg of protein) groups were also higher (*P*<0.001) than the values observed in anestrus (0.35±0.06nM/mg of protein). These results indicate modulation of insulin binding characteristics during different phases of the estrous cycle in dogs, showing that muscle insulin binding affinity for its receptor is reduced during estrus and diestrus. However, this poor hormone-receptor affinity is compensated for by a greater total binding capacity, once there is no difference in patients' glycemic response after an intravenous glucose load.

INDEX TERMS: Muscle tissue, insulin sensitivity, tyrosine kinase receptors, insulin resistance, estrous cycle, diabetes mellitus, dogs.

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<sup>2</sup> Setor de Clínica de Pequenos Animais, Hospital de Clínicas Veterinárias. Departamento de Medicina Animal, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Avenida Bento Gonçalves 9090, Agronomia, Porto Alegre, RS 91540-000, Brazil. \*Corresponding author: [alan.poppl@ufrgs.br](mailto:alan.poppl@ufrgs.br)

<sup>3</sup> Laboratório de Metabolismo e Endocrinologia Comparada, Departamento de Fisiologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Sarmiento Leite 500, Porto Alegre, RS 90050-170, Brazil.

<sup>4</sup> Laboratório de Análises Clínicas Veterinárias, Hospital de Clínicas Veterinárias, Departamento de Patologia Clínica Animal, Faculdade de Veterinária, UFRGS, Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil.

**RESUMO.- [Características de ligação da insulina no tecido muscular canino: efeitos da fase do ciclo estral.]** As flutuações hormonais durante as diferentes fases do ciclo estral são uma causa importante de resistência insulínica em fêmeas caninas, e poucas informações são conhecidas sobre defeitos na ligação da insulina ao seu receptor, ou defeitos pós-receptor associados com resistência à insulina em cães. Para avaliar as características da ligação insulina-receptor no tecido muscular de cadelas durante o ciclo estral, dezessete pacientes foram utilizadas no estudo (seis em anestro, cinco em estro e seis em diestro). Um teste de tolerância à glicose intravenosa (IVGTT) foi realizado em todas as pacientes por meio da infusão de 1mL/kg de uma solução de glicose 50% (500mg/kg), com coletas de sangue para determinação de glicemia nos tempos 0, 3, 5, 7, 15, 30, 45 e 60 minutos da injeção de glicose. Amostras de tecido muscular foram coletadas durante ovariectomia, imediatamente congeladas em nitrogênio líquido, e posteriormente armazenadas a -80°C até a preparação das membranas por meio de homogeneização e centrifugação sequencial. Para os experimentos de ligação hormônio-receptor, as membranas foram incubadas na presença de 20.000cpm de <sup>125</sup>I-insulina humana, e concentrações crescentes de insulina regular humana não marcada para saturação fria. O IVGTT não mostrou diferenças entre as pacientes em diferentes fases do ciclo estral com relação a glicemia basal, ou na resposta glicêmica após infusão de glicose nos tempos estudados. Dois sítios de ligação da insulina, um de alta-afinidade, e outro de baixa afinidade, foram detectados pela análise de Scatchard, e diferenças significativas foram detectadas na constante de dissociação (Kd1) e capacidade de ligação máxima (Bmax1) dos sítios de ligação de alta-afinidade. O Kd1 para o grupo anestro (6,54±2,77nM/mg de proteína) foi menor ( $P<0,001$ ) que os Kd1 dos grupos estro (28,54±6,94 nM/mg de proteína) e diestro (15,56±3,88nM/mg de proteína). Os Bmax1 dos grupos estro (0,83±0,42nM/mg de proteína) e diestro (1,24±0,24nM/mg de proteína) também foram maiores que os valores encontrados no grupo anestro (0,35±0,06nM/mg de proteína). Estes resultados demonstram uma modulação das características de ligação da insulina nas diferentes fases do ciclo estral em cães, evidenciando uma menor afinidade de ligação da insulina ao seu receptor no tecido muscular durante o estro e diestro. Contudo, esta menor afinidade de ligação hormônio-receptor é compensada por uma maior capacidade de ligação, o que fica também evidenciado pela ausência de diferenças na resposta glicêmica das pacientes após um desafio com glicose por via endovenosa.

**TERMOS DE INDEXAÇÃO:** Músculo, caninos, sensibilidade insulínica, receptores tirosina quinase, resistência insulínica, ciclo estral, diabetes mellitus.

## INTRODUCTION

The insulin receptor (IR) belongs to a subfamily of tyrosine kinase receptors, and so do the type-I insulin-like growth factor (IGF-IR) and the orphan insulin receptor-related receptor (IRR) (Lawrence et al. 2007). Moreover, IR/IGF-

-IR hybrids have been described in all tissues that express both receptors and, despite low affinity, insulin and insulin-like growth factor-1 can bind each other's receptors due to their strong structural similarities (Dominici et al. 2005). Those receptors are a transmembrane tetrameric protein that consists of two  $\alpha$ - and two  $\beta$ -subunits. Insulin binding to the extracellular monomeric  $\alpha$ -subunits leads to the intracellular activation of the intrinsic kinase activity of the  $\beta$ -subunits (Saltiel & Kahn, 2001). Two ligand binding sites are often recognized in each  $\alpha$ -subunit monomer due to their curvilinear Scatchard plots, and negative cooperativity: the low-affinity (S1) and the high-affinity (S2) binding sites (Schäffer 1994).

The origin of many insulin-resistant clinical conditions may be seated on reduced tissue sensitivity to insulin due to a receptor or a post-receptor defect (Feldman & Nelson 2004, Hess 2009). In this scenario, insulin binding studies are useful for evaluating tissue insulin sensitivity (Johnston et al. 1991). In dogs, the most common physiological cause of insulin resistance is described in the female dog during the hormonal fluctuations of the estrous cycle. During diestrus, progestogens induce the synthesis and release of growth hormone (GH) by the mammary glands in a paracrine and endocrine fashion, potentially triggering diabetes mellitus (Eingenmann et al. 1983, Selman et al. 1994). Moreover, Ryan & Enns (1988) demonstrated regulation of insulin binding capacity and post-binding function by estradiol and progesterone *in vitro*. Since little is known about the defects at IR or post IR levels in dogs (Pöpl et al. 2012), the objective of this paper was to study canine insulin binding properties in muscle tissue of bitches in anestrous, estrus, and diestrus.

## MATERIALS AND METHODS

This study was approved by the Research Ethics Committee of the University (CEP/UFRGS) and by the Research Committee of the Basic Health Sciences Institute (COMPESQ/ICBS). Permission for the participation of animals in the study was obtained from their owners through a signed informed consent form.

Sixteen owned bitches brought for spaying surgery were evaluated at the Veterinary Clinical Hospital of the Federal University of Rio Grande do Sul, Brazil (HCV/UFRGS). The animals were split into three groups: anestrus ( $n=6$ ), estrus ( $n=5$ ), and diestrus ( $n=6$ ) according to reproductive history, vaginal cytology, and uterine histology after surgery. The combination of vaginal smears and uterine histology was adopted as gold standard for the classification of the estrous cycle phase (Galabova et al. 2003, Feldman & Nelson 2004). The inclusion criteria were as follows: weight greater than 6 kg, normal complete blood count (CBC), absence of diseases or use of medications, ideal body condition (Laflamme 1997) and no exposure to exogenous progestogens or estrogens in the past 6 months. All animals were fed commercial food by their owners.

Prior to surgery, the bitches were submitted to an intravenous glucose tolerance test as described by Mattheeuws et al. (1984). After overnight fasting, the animals were admitted to the hospital. A catheter was placed in each cephalic vein for glucose determinations and glucose infusion, respectively. After baseline blood sampling for glucose determination, an intravenous injection of 1mL/kg of a glucose 50% solution (500 mg/kg) was made through the second catheter within 30 seconds. The catheter for

blood sampling was flushed with heparin for further collection of blood samples for glucose determination at 0, 3, 5, 7, 15, 30, 45 and 60 minutes after glucose infusion. Plasma glucose was determined by the glucose oxidase method using an enzymatic colorimetric assay.

After being starved for 8 to 12 h, the patients were anesthetized with 3 mg/kg of IM meperidine (Dolosal, Cristália) followed by 5mg/kg of IV propofol (Provine, Claris), according to the standard protocol, and maintained with orotracheal intubation with 2% isoflurane (Forane, Abbot) in oxygen (White Martins). After spaying, 1-g samples of the rectoabdominal muscle were collected from each animal, and were immediately frozen in liquid nitrogen, and then stored at -80°C until the membranes were prepared as recently described by Pöppel et al. (2012). All patients received postoperative care in compliance with the hospital's standard guidelines.

The muscle tissues were homogenized by an Ultra-Turrax® homogenizer at 4°C in a buffer containing phenylmethylsulfonyl fluoride (PMSF) 100mM, TRIS 10mM, EDTA 1mM, and sucrose 250mM, pH 7.4 (TES buffer). The muscle membranes were prepared by repeated centrifugation (5 min at 3000xg, at 4°C, followed by 20 min at 30000xg of the supernatant), and the pellets were resuspended in 250-350µL of buffer. To dissociate the membrane hormone-receptors complex sometimes present in these pellets (Turyn et al. 1986), after resuspended, the membranes were incubated (5 min, at 25°C) in acetic acid 1mM using a 1/1 (v/v) ratio. The reaction was interrupted with TES buffer at a 2/1 (v/v) ratio and re-centrifuged (30 min at 30000xg, at 4°C). The final pellets were resuspended again in 250-350µL of TES buffer with PMSF. The protein content of the samples was measured according to Bradford (1976) using bovine serum albumin as standard.

Binding experiments were performed according to Kucharski et al. (1997). Four hundred micrograms of membrane proteins were used per tube. Membrane proteins were incubated with Mammalian Krebs-Ringer buffer (MKR) 1% BSA, pH 7.4, in the presence of increasing concentrations of human regular insulin (Eli Lilly - 0.1µg/ml, 1µg/ml, 10µg/ml, 100µg/ml, 250µg/ml and 500µg/ml) plus 20,000cpm of human <sup>125</sup>I-insulin (Amersham Biosciences - 2.000 Ci/mmol). For total binding determination, membrane proteins were incubated only with <sup>125</sup>I-insulin. The studies were performed in duplicate for each animal. After 2 hours of incubation at 25°C, the contents of the tubes were filtered in a glass microfiber filter (Whatman GF/B 2.4cm). Each filter was rinsed five times with 1 ml of MKR 0.1% BSA. After dried, the formation of <sup>125</sup>I-insulin/receptor complexes present in the filters was measured in an LKB counter. Insulin competition curves (cold saturation) were analyzed by Kell for Windows™ version 6 (Biosoft), for generation of the Scatchard plots, dissociation constants (Kd), as well as total binding capacity (Bmax).

The IVGTT results were compared among groups by repeated measures ANOVA while Kd and Bmax results were expressed as mean (±) standard deviation. A one-way ANOVA was applied, followed by Tukey's test, to compare the results obtained from the anestrus, estrus, and diestrus groups. The analyses were performed in SAS (Statistical Analysis System version 9.1).

**RESULTS**

No significant difference was detected (*P*>0.05) between age and weight in the different groups (Table 1). The IVGTT showed no differences among bitches during the estrous cycle regarding baseline glycemia, or glycemic response after glucose infusion (Fig.1). Binding data indicate

the presence of two insulin binding sites: one with high affinity / low capacity and one with low affinity/high capacity. Statistical differences were observed in dissociation constants (*Kd*1) and total binding capacities (*Bmax*1) of the high-affinity binding sites. *Kd*1 for the anestrus group was 6.54±2.77nM/mg of protein, but it was significantly higher (*P*<0.001) for the estrus (28.54±6.94nM/mg of protein) and diestrus (15.56±3.88nM/mg of protein) groups. *Bmax*1 for the estrus (0.83±0.42nM/mg of protein) and diestrus (1.24±0.24nM/mg of protein) groups were also higher (*P*<0.001) than the values observed in the anestrus group (0.35±0.06nM/mg of protein). However, *Kd*2 and *Bmax*2 for the low-affinity binding sites did not differ (*P*>0.05) among groups (Table 2). Representative Scatchard plots for animals in anestrus, estrus, and diestrus are shown in Figure 2 and were drafted as the

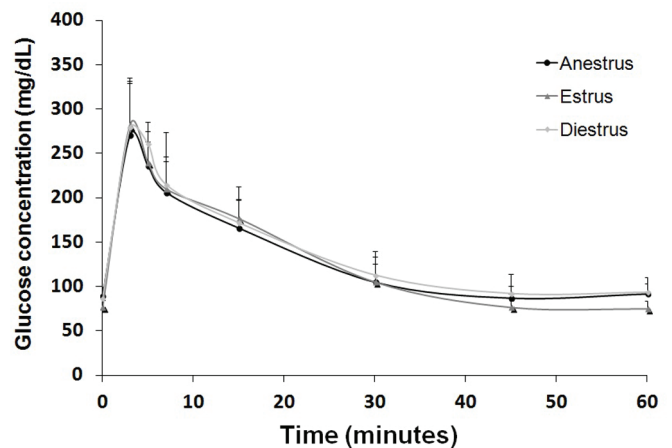


Fig.1. Intravenous glucose tolerance test in bitches showing no differences in baseline glycemia or glycemic response after glucose infusion. Each point on the curves represents the mean with standard deviation bars.

**Table 1. Mean ± SD and range values for age (years) and body weight (kg) in bitches in different phases of the estrous cycle**

	Age (years)	Body weight (Kg)
Anestrus (n = 6)	1.59 ± 0.52 (1-2) <sup>a</sup>	16.7 ± 5.24 (7.5-29) <sup>b</sup>
Estrus (n = 5)	2.34 ± 1.15 (1.3-2.5) <sup>a</sup>	14.9 ± 6.33 (6-29) <sup>b</sup>
Diestrus (n = 6)	3.15 ± 2.54 (1.8-10) <sup>a</sup>	18.3 ± 7.68 (6.1-26.6) <sup>b</sup>

<sup>a</sup> There was no difference among each phase in age, <sup>b</sup> body weight (*p*>0.05).

**Table 2. Characteristics of insulin binding in canine rectoabdominal muscle during estrous cycle**

	<i>Kd</i> 1* (nM/mg ptn)	<i>Bmax</i> 1† (nM/mg ptn)	<i>Kd</i> 2** (pM/mg ptn)	<i>Bmax</i> 2‡ (pM/mg ptn)
Anestrus	6.54 ± 2.77 <sup>a</sup>	0.35 ± 0.06 <sup>a</sup>	26.06 ± 22.29 <sup>d</sup>	0.79 ± 0.52 <sup>d</sup>
Estrus	28.54 ± 6.94 <sup>b</sup>	0.83 ± 0.42 <sup>b</sup>	25.68 ± 12.79 <sup>d</sup>	0.35 ± 0.2 <sup>d</sup>
Diestrus	15.56 ± 3.88 <sup>c</sup>	1.24 ± 0.24 <sup>b</sup>	18.56 ± 7.36 <sup>d</sup>	0.77 ± 0.37 <sup>d</sup>

\* Dissociation constant for the high-affinity binding site, † Maximal binding capacity for the high-affinity binding site, \*\* Dissociation constant for the low-affinity binding site, ‡ Maximal binding capacity for the low-affinity binding site. The *Kd* and *Bmax* results were expressed as nM per mg of protein as the mean ± SD. Different letters (<sup>a,b,c</sup>) represent significantly difference (*p*<0.001) among groups by one-way analysis of variance followed by Tukey's test.

bound to free insulin ratio against the bound insulin concentration (pM) per milligram of protein. Figure 3 shows the competitive inhibition curves of  $^{125}\text{I}$ -insulin binding to muscle membranes by increasing cold human insulin concentrations (cold saturation) for the different estrous cycle phases.

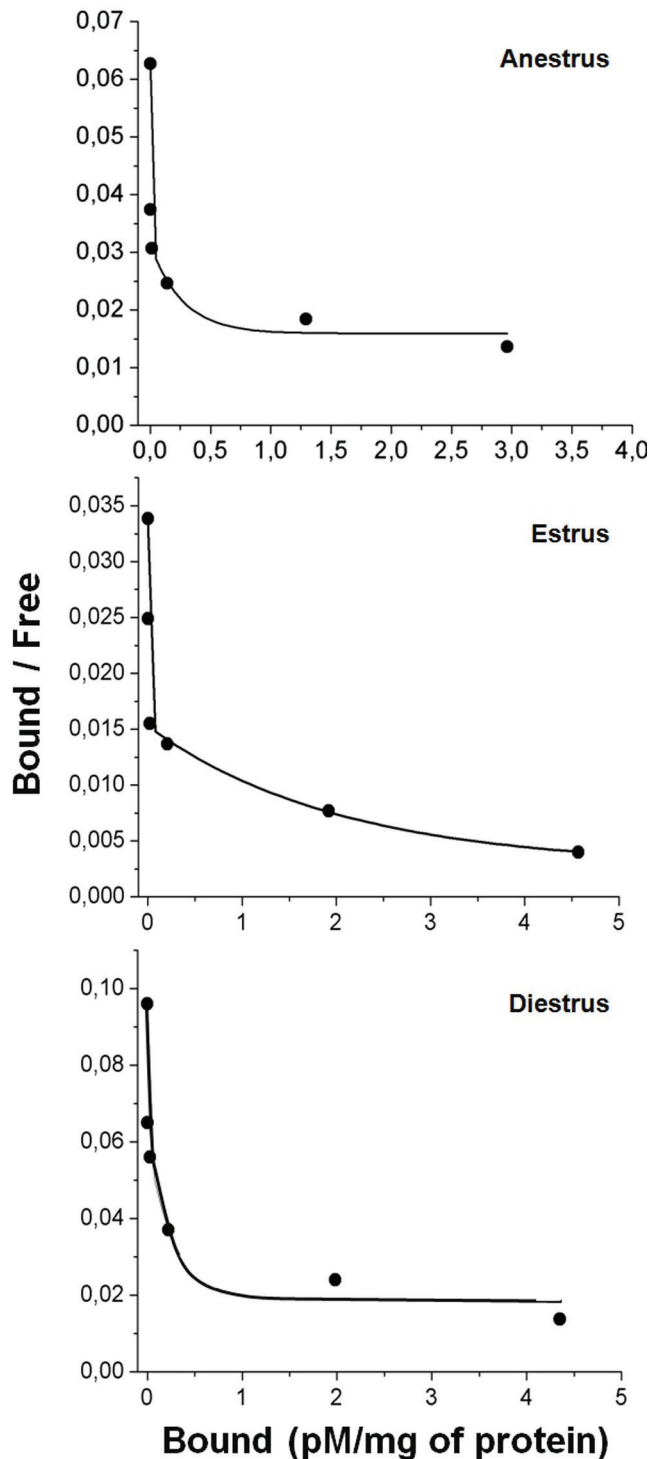


Fig.2. Representative Scatchard plots for bitches during estrous cycle drafted as the bound to free insulin ratio against the bound insulin concentration (pM) per milligram of protein. The curvilinear relationship reveals two insulin binding sites.

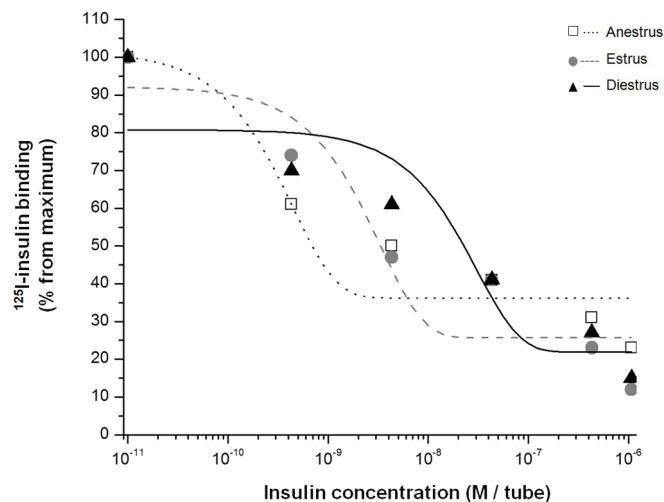


Fig.3. Cold saturation curves for anestrus, estrus, and diestrus. Incubation of membranes with increasing quantities of unlabeled human insulin (x axis) causes reduction in specifically  $^{125}\text{I}$ -insulin bound (y axis), so the further to the right the curve is, the greater the insulin resistance. These data were inserted in the Ligand sub-directory of Kell for Windows™ version 6 (Biosoft) to generate  $K_d$  and  $B_{max}$  values for both insulin binding sites shown in Table 2.

## DISCUSSION

The detection of two insulin binding sites in canine muscle is in agreement with insulin binding data previously described for dogs by Johnston et al. (1991). It was proposed that binding of insulin to the S1 of one  $\alpha$ -monomer is followed by cross-linking of insulin to the S2' of the alternate  $\alpha$ -monomer, with resultant S1/S2' high-affinity binding. Negative cooperativity results from subsequent insulin binding to the alternate S1'/S2 pair and from the concomitant release of insulin from the S1/S2' region, once a pair of ligands cannot cross-link. The presence of the second insulin molecule bound to the S2 prevents the formation of an alternate high-affinity S1'/S2 cross-link through steric interference and thus the accelerated dissociation of the pre-bound insulin at the S1/S2' site (Lawrence et al. 2007).

The interaction between insulin and IR is reversible, and when a system reaches an equilibrium, for each insulin-IR complex formed, another insulin-IR complex dissociates at the same rate. The dissociation constant ( $K_d$ ) is then considered to be the inverse association constant ( $K_a$ ), and represents the insulin-free concentration needed to saturate half of the insulin receptors in the system (Sanvito et al. 1994), or in this case, the cold insulin concentration needed to reduce maximal  $^{125}\text{I}$ -insulin binding by 50% (Johnston et al. 1991). Therefore,  $K_d$  is assumed as a measure of tissue insulin sensitivity, and the higher the  $K_d$ , the smaller the insulin sensitivity.

The analysis of our data concerning insulin binding characteristics during estrous cycle clearly shows that the muscle tissue became more insulin-resistant during estrus and diestrus due to elevated  $K_d$  values. However, the maximum insulin binding capacity during these estrous cycle phases has increased and seems to compensate for the lower binding affinity (Table 2). These changes in insulin bin-

ding characteristics were detected only at the high-affinity binding sites, and it seems that in dogs, low-affinity binding sites are less susceptible to modulation, once Johnston et al. (1991), after studying insulin binding properties in different tissues of adult and newborn dogs, also found changes only at high-affinity binding sites.

The compensation for reduced binding affinity by means of the increase in binding capacity apparently was achieved in this population, once no differences were found in baseline glucose levels or glycemic response during the IVGTT. We previously studied different insulin sensitivity indexes in the cohort from which the bitches of this study were selected, and despite higher values for different insulin sensitivity indexes during estrus and diestrus, no significant statistical difference was found (Pöppl et al., 2009). Nevertheless, Fukuta et al. (2012), using the euglycemic-hyperinsulinemic glucose clamp, demonstrated a 40% reduction in insulin sensitivity in bitches in diestrus despite no differences in baseline insulin or baseline glucose plasma concentrations. Moreover, Mared et al. (2012) in a longitudinal study of Elkhounds during anestrus and diestrus documented higher C-peptide plasma concentration and HOMA-B, and a lower HOMA-S during diestrus (and higher progesterone concentration), despite no difference in GH or IGF-I levels.

The GH-related insulin resistance observed during diestrus is more likely to be detected in older bitches (Rijnberk et al. 2003), and for the younger ones, insulin resistance documented during exposure to progestogens may be related to modulation of tissue insulin sensitivity in binding or post-binding steps (Pöppl et al. 2012). In women with gestational diabetes mellitus, however, the problem regarding tissue insulin sensitivity is restricted to post-binding steps, such as reduced tyrosine kinase activity, decreased expression of insulin receptor substrate-1 (IRS-1) and increased levels of the p85 $\alpha$  subunit of PI 3-kinase (Barbour et al. 2007). Interestingly, estrogens have been implicated in changes in insulin secretion, and also in reduced insulin sensitivity and glucose tolerance, especially in muscles (Godsland 2005, Barros et al. 2006). Batista et al. (2005) showed that chronic exposure of bitches to estradiol and progesterone causes insulin resistance in the whole body, but primarily in the skeletal muscle. Notwithstanding, little is known about the effects of estrogens on insulin sensitivity in dogs, but reduced baseline tyrosine kinase activity was reported recently in muscle tissue during estrous (Pöppl et al. 2012) and higher *K<sub>d</sub>* values were observed in bitches in estrous in this study. As a matter of fact, in bitches, progesterone plasma concentration starts to increase during proestrus (Concannon et al. 1989).

## CONCLUSIONS

Therefore, we conclude that muscle insulin sensitivity may be modulated in an insulin binding step during the estrous cycle in bitches, and despite the fact that the mechanisms that underlie these changes in insulin sensitivity and maximum binding capacity are still unclear, complex hormonal changes during the estrous cycle are likely involved,

explaining the larger incidence of diabetes mellitus during estrus and diestrus in bitches.

Further molecular studies are needed for the better understanding of these hormonal relationships with insulin sensitivity in dogs during the estrous cycle.

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