

## Original article

**Heterogeneity of circulating prolactin in the bitch**

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**Abstract**—Different molecular forms of circulating prolactin (PRL) are known to occur in several species. As no such information was available in dogs, we assessed the molecular profile of circulating PRL in bitches. Pooled sera from covertly (CTRL) and overtly pseudopregnant (PSPT) diestrous bitches with high or low ( $> 10$  or  $< 10$  ng·mL<sup>-1</sup>, respectively) serum PRL (measured by ELISA) were analyzed by Sephadex G–100 and Concanavalin A–Sephadex column chromatography. Four serum PRL fractions were identified and termed big-big, big ( $> 67$  kDa), native (23 kDa) and fragmented ( $< 20$  kDa) PRL. The percentages of these fractions were roughly similar in CTRL and PSPT animals, irrespective of their serum PRL levels (higher in PSPT than in CTRL bitches). A large proportion of glycosylated PRL (between 69 and 100%) was also detected in these sera. We conclude that in dogs, circulating PRL occurs in multiple molecular forms, whose relative abundance is comparable in covertly and overtly pseudopregnant bitches.

**prolactin / molecular variants / bitch / diestrus / pseudopregnancy**

**Résumé**—**Hétérogénéité de la prolactine circulante chez la chienne.** La prolactine (PRL) circulante se trouve, dans différentes espèces, sous plusieurs formes moléculaires. Compte tenu du fait qu'il n'existe pas d'information disponible chez le chien, nous avons décidé d'étudier le profil moléculaire de la PRL circulante chez cet animal. Nous avons utilisé des pools de sérums provenant de chiennes normales en diestrus (CTRL) ou pseudogestantes (PSPT), ayant des taux élevés ou bas de PRL ( $> 10$  ou  $< 10$  ng·mL<sup>-1</sup> respectivement; dosages effectués par Elisa). Les sérums ont été analysés sur colonne de Séphadex G–100 et par chromatographie d'affinité, sur colonne de Séphadex couplée à de la Concanavoline A. La chromatographie sur G–100 a identifié quatre fractions de PRL, qui ont

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été appelées big-big, big (PM < 67 kDa), native (23 kDa) et fragmentée (< 20 kDa). Les pourcentages relatifs de ces fractions sont identiques chez les CTRL et les PSPT, quel que soit le taux, élevé ou bas, de PRL sérique (plus haut chez les animaux CTRL). De plus, une proportion importante de PRL sérique (69 à 100 %) est sous forme glycosylée. En conclusion, la PRL sérique se trouve, chez le chien, sous des formes moléculaires multiples, dont l'abondance relative est comparable chez la chienne normale et pseudogestante.

## **prolactine / variant moléculaire / chienne / diestrus / pseudogestation**

### **1. INTRODUCTION**

Prolactin (PRL) is known to be a 197–198 amino acid peptide with a mass of 23–24 kDa. Research has uncovered a surprising degree of molecular heterogeneity for circulating PRL, with different biopotencies associated with varying molecular forms of the hormone [24, 25]. Thus, small molecular weight PRL forms have been described, which may result from alternative splicing of the PRL gene's primary transcript or proteolytic cleavage, which gives rise to fragments of 22, 21, 17, 16 and 5.8 kDa [25]. While the 17 and 16 kDa variants exhibit a biopotency to immunoreactivity ratio similar to that of intact PRL, the biological activity of 22 kDa PRL remains to be investigated [25]. In an initial study, a 16 kDa fragment was found to have a greater mammary mitogenic activity than intact PRL [18] but in a later report the opposite was found in rats [3]. On the other hand, high molecular weight forms of PRL were detected in the pituitary gland and plasma of most species examined [14, 27]. They have been referred to as big-big (MW > 60 kDa) and big (MW ≈ 45 kDa). The physiological significance of these larger forms is unknown because, in general, their biopotency lower than that of native PRL [24, 26].

In the bitch, there is no documented information about the molecular profiles of circulating PRL in any stage of the estrous cycle. Certainly, the possibility exists that PRL variants may also occur in dogs. If so,

eventual differences in the proportion of PRL forms (each of them endowed with different bioactivity/immunoreactivity ratios) among bitches, could explain, at least in part, the lack of consistency found in some bitches, between immunoassayable circulating levels of PRL and the intensity of symptoms in some PRL-associated syndromes, like pseudopregnancy [10–12, 15, 20, 22].

It was, therefore, of interest to determine whether in the bitch, PRL circulates as a single molecular entity or as multiple molecular variants. A second point of interest was to compare the molecular profiles of serum PRL between covert and overt pseudopregnant bitches. Since the circulating concentration levels of both PRL and progesterone ( $P_4$ ) have been implicated in the etiology of pseudopregnancy [5–8], the serum concentrations of these hormones were compared between these two groups.

### **2. MATERIALS AND METHODS**

#### **2.1. Animals**

Fifty-five privately owned cross- and pure-bred bitches aged 2 to 10 years and weighing 7 to 30 kg were used. They all had had their previous estrus between 8 and 12 weeks prior to the commencement of the study. The bitches were divided into 2 groups, taking into account the presence of overt pseudopregnancy (pseudopregnant

group [PSPT],  $n = 30$ ) or absence of symptoms (covert pseudopregnancy; control group [CTRL],  $n = 25$ ). Overt pseudopregnancy was diagnosed based on the presence of mammary enlargement with milk secretion, which was frequently associated with maternal behavior [6, 7].

## 2.2. Blood sampling

Blood samples for PRL and  $P_4$  determination were collected by peripheral (jugular/cephalic/saphenous) venepuncture from 9 and 11 am. Samples were centrifuged at 4000 *g* for 15 min, serum obtained and stored at  $-20\text{ }^\circ\text{C}$  until hormone assays were performed.

## 2.3. Hormone assays

Serum PRL was measured by a homologous end point enzymoimmunoassay (Milenia, DPC<sup>®</sup>, Bad Nauheim, Germany). The intra- and inter-assay coefficients of variation were 3.5 and 9.4%, respectively. The lowest limit of detection at 95% binding (sensitivity) was  $0.5\text{ ng}\cdot\text{mL}^{-1}$ . Serum  $P_4$  was measured by radioimmunoassay, using a solid phase kit (Coat-A-Count, DPC<sup>®</sup>, Los Angeles, USA). For this kit, the sensitivity at 95% binding was  $0.1\text{ ng}\cdot\text{mL}^{-1}$  and the intra- and inter-assay coefficients of variation were 3.8 and 4.5%, respectively.

## 2.4. Gel chromatography

A total of 47 serum samples were used for chromatographic analysis. Samples were grouped according to the clinical status of animals (CTRL = C or PSPT = P) and their serum PRL levels (high PRL (H)  $>$  or low PRL (L)  $< 10\text{ ng}\cdot\text{mL}^{-1}$ ). Within each group, serum pools were prepared by mixing 300  $\mu\text{L}$  of each sample. The pools so generated were as follows:

– P-H: pooled sera from PSPT animals with high PRL levels ( $n = 13$ ).

– P-L: pooled sera from PSPT animals with low PRL levels ( $n = 9$ ).

– C-H: pooled sera from CTRL animals with high PRL levels ( $n = 5$ ).

– C-L: pooled sera from CTRL animals with low PRL levels ( $n = 20$ ).

The serum pools were chromatographed on a Sephadex G-100 column ( $100 \times 1.6\text{ cm}$ ; Pharmacia, Uppsala, Sweden) equilibrated at  $20\text{ }^\circ\text{C}$  with 0.05 M phosphate-buffered saline, pH 7.4, containing 0.2% bovine serum albumin (BSA; Sigma, Saint-Louis, Missouri). The same buffer was used for elution. The column was calibrated with dextran blue (MW = 2000 kDa), BSA (MW = 67 kDa) and  $^{125}\text{I}$ -hPRL (MW = 23 kDa) as previously described [14]. Four-ml serum aliquots were loaded, in separate runs, onto the column and buffer was run through by means of a peristaltic pump at a flow rate of approximately  $7.5\text{ mL}\cdot\text{h}^{-1}$ . A total of 72 fractions of 2 mL each were collected per run and were frozen until PRL determination.

## 2.5. Affinity chromatography

In order to separate glycosylated PRL forms, 1 mL sample was applied to a column of concanavalin A-Sepharose ( $100 \times 2.5\text{ cm}$ ; Pharmacia, Uppsala). The concanavalin A-adsorbed material was eluted with 0.2 M methyl-D mannopyranoside (Sigma, Saint-Louis, Missouri) as previously described [14]. Samples were kept frozen until PRL determination.

## 2.6. Statistical analysis

Data were analysed by least-squares means (LSM) analysis of the variance using the General Linear Model procedure (PROC GLM, SAS<sup>®</sup> [23]) for serum PRL and  $P_4$ , the percentage of PRL variants and of G-PRL forms. The mathematical model included the main effect of group (PSPT/CTRL). The level of significance was set at 0.05.

### 3. RESULTS

#### 3.1. Chromatographic analysis of bitch sera

Gel chromatography revealed the presence of multiple molecular forms of immunoassayable PRL in bitch serum (Fig. 1). This molecular heterogeneity occurred in both CTRL and PSPT animals, irrespective of their serum PRL levels. Four predominant molecular forms were identified. By analogy with human PRL, the four fractions were tentatively referred to as, big-big, big, native and fragmented, from the largest to the smallest. Big-big was larger than the BSA marker, whereas big was slightly smaller than BSA (67 kDa). The PRL fraction termed native had a molecular size very close to that of the hPRL marker (23 kDa) while the fragmented form was smaller than 20 kDa. The percentage of the big-big and big forms as well as the peaks probably associated with native and fragmented hormone were variable in the different serum pools (Fig. 2). However, no clear differences in relative abundance were

evident among groups for any of the different PRL variants.

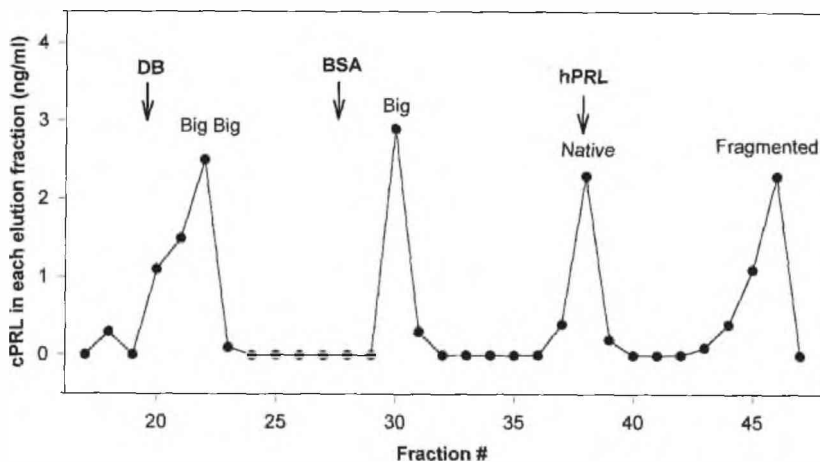
A large proportion of glycosylated PRL was also detected. It constituted 69, 77, 70 and 100% of total PRL for the P-H, P-L, C-H and C-L groups, respectively.

#### 3.2. Serum PRL levels in CTRL and PSPT bitches

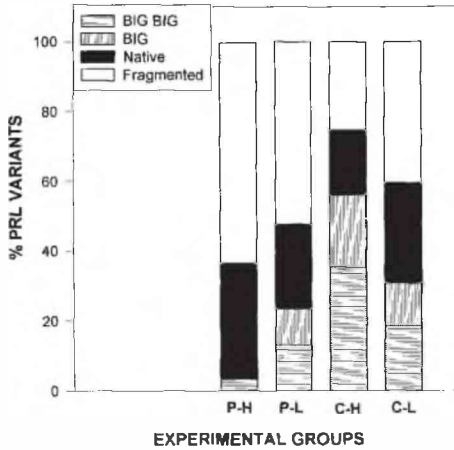
Serum PRL concentrations were significantly higher in PSPT ( $13.8 \pm 2.6$  ng·mL<sup>-1</sup>, range 0.5 to 80 ng·mL<sup>-1</sup>) than in CTRL bitches ( $6.3 \pm 1.6$  ng·mL<sup>-1</sup>, range 0.5 to 37.7 ng·mL<sup>-1</sup>;  $p = 0.02$  (Fig. 3). On the other hand, no significant differences were found in P<sub>4</sub> concentrations between PSPT ( $1.3 \pm 0.3$  ng·mL<sup>-1</sup>) and CTRL ( $1.1 \pm 0.3$  ng·mL<sup>-1</sup>;  $p = 0.79$ ).

### 4. DISCUSSION

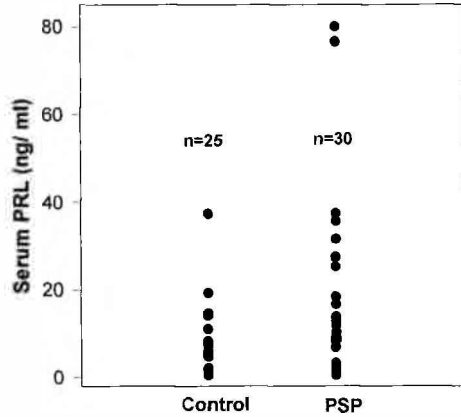
Our results document, for the first time, that in the dog there are different molecular



**Figure 1.** Chromatographic profile for serum PRL in bitches. The chromatogram shown represents a typical PRL profile observed in the serum of bitches. This example corresponds to a serum pool from C-H animals. The sample was run on a Sephadex G-100 column. Pool volume seeded was 4 mL. The names given to the different PRL peaks were assigned by analogy with known human PRL variants of roughly similar sizes. Markers: DB = dextran blue, MW 2000 kDa; BSA = bovine serum albumin, MW 67 kDa; hPRL = <sup>125</sup>I-hPRL, MW 23 kDa. For further details see the text.



**Figure 2.** Distribution of PRL variants in the different serum pools. Percentages refer to the sum of all peaks for each serum pool. P-H: PSP with high PRL levels; P-L: PSP with low PRL; C-H: CTRL with high PRL; C-L: CTRL with low PRL. For further details see the text.



**Figure 3.** Serum PRL levels in diestrous bitches. Covert (CTRL) and overt PSPT bitches were bled under comparable experimental conditions.

forms of circulating PRL, thus enlarging the list of species known to possess multiple molecular PRL forms in the circulation. Nevertheless, the present results should be regarded as an initial study, where no extensive biochemical or biological characterization of the different fractions was attempted. Since in other species circulating PRL is known to occur in multiple molecular forms, each of them endowed with different bioactivity levels, it would be of interest to characterize the bioactivity to immunoreactivity ratio of the four PRL variants described here.

Although PRL was long regarded as a non glycoprotein hormone, it is now known that a proportion of PRL molecules is actually glycosylated, with a degree of glycosylation that varies among species, ranging from a very low level to more than 50%. Thus, in humans glycosylated PRL (G-PRL) has been reported to be up to 20, 32 and 50% in plasma in different studies [2, 4, 16] while in the same biologic fluid from rats it was reported to be 60% [1]. The biological activity of G-PRL in some of the classi-

cal tests for the hormone, such as pigeon crop sac stimulation, Nb2 lymphoma cell proliferation and mammary casein synthesis is similar to or lower than non glycosylated PRL [16]. However, some later studies claimed greater than normal pigeon crop sac stimulating activity for porcine G-PRL [21]. Receptor binding activity and immunologic cross-reactivity are also greatly reduced as a result of glycosylation, thus it has been suggested that glycosylation may down regulate PRL action at target tissues [25]. Glycosylation seems to facilitate proteolytic cleavage of the molecule to a 17 kDa form, and thus may serve a role in regulation of the processing of PRL [25]. In this context, it is of interest that our bitches displayed a higher proportion of circulating G-PRL than most species studied so far. Whether this high degree of glycosylation of canine PRL plays any physiological role, remains to be investigated.

Our finding of significantly higher PRL levels in PSPT than in CTRL bitches is in agreement with some previous reports [9, 19], although a number of other studies have

not detected significant differences in PRL levels between overt and covert PSPT bitches [11, 12, 15, 20, 22]. Indeed, a universal serum PRL threshold for triggering pseudopregnancy in the bitch is unlikely to exist. This is in line with studies in women where, in some individuals, no correlation was detected between typical signs of hyperprolactinemia and serum PRL concentrations [13, 14, 27].

The present study originated from our interest to elucidate a paradox frequently found in bitches: While PRL is generally accepted to play a key role in the genesis of pseudopregnancy in diestrous bitches [7, 9–11, 17, 19, 22], it is a fairly frequent finding that animals with high (immunoassayable) PRL levels do not develop overt pseudopregnancy symptoms while others with lower serum PRL do (this can be appreciated in Fig. 3). We reasoned that if in the bitch, PRL circulated under multiple molecular forms of varying bioactivity, then overt PSPT animals with low immunoassayable serum PRL could possess a prevalence of PRL variants with high bioactivity to immunoreactivity ratio, with the opposite happening in covert PSPT animals with relatively high PRL levels. The present results confirm the first part of our hypothesis; that is, canine PRL circulates under multiple molecular forms. However, the data reveal a rough similarity in the distribution of PRL forms in PSPT and CTRL bitches, irrespective of their serum PRL levels, thus failing to support the second part of our hypothesis.

We conclude that in dogs, circulating PRL occurs in multiple molecular forms and that the relative abundance of these forms in bitches is comparable between bitches with and without overt pseudopregnancy.

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