IGF system and ovarian folliculogenesis in dog breeds of various sizes: is there a link?

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Abstract

The IGF system plays a crucial role in ovarian folliculogenesis, and changes in IGF-binding protein (IGFBP) levels modulate IGF bioavailability. Data from various mammalian models suggest a link between body size, IGF1 in serum and female reproduction parameters. Among the vertebrate species, the dog exhibits the widest span in body height. Height is known to be positively correlated with the concentration of serum IGF1. In this work, the ovarian physiology of 40 bitches exhibiting a wide span of height, and breed type was investigated. IGF1, IGF2, IGFBP3, estradiol (E₂), and progesterone concentrations in plasma and preovulatory follicular fluid were quantified. A total of 455 follicles, 2–8 mm in diameter, were recovered at the preovulatory stage, measured, and punctured. Intrafollicular levels of IGF1 were positively correlated with plasma levels, and plasma IGF1 levels were positively correlated with both

bitch height and weight. The concentrations were threefold higher in large dogs compared with small dogs. A positive correlation between intrafollicular and plasmatic IGFBP3 levels and a positive correlation between plasmatic IGFBP3 levels, and both height and weight of the bitches were observed. The number of preovulatory follicles and the diameter of the three largest follicles were positively correlated with bitch height. E₂ intrafollicular concentrations were higher in preovulatory follicles from small animals than in those from large animals. In conclusion, the strong variability in height between dogs appeared to be associated with dramatic differences in IGF1, and IGFBP3 levels, in both plasma and follicular fluid. These differences were associated with significant differences in some functional aspects of ovarian follicles.

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Introduction

The insulin-like growth factor (IGF) system has been shown to play a key role in ovarian folliculogenesis (Mazerbourg *et al.* 2003). IGF1 stimulates granulosa cell proliferation and enhances the biological effects of FSH and LH on granulosa and thecal cells. In particular, IGF1 is a potent stimulator of steroidogenesis by ovarian cells. IGF1 intrafollicular concentrations hardly change at all during the last stages of folliculogenesis in large growing antral follicles (for review see Spicer & Echternkamp (1995) and Mazerbourg *et al.* (2003)). Rather, IGF bioavailability dramatically increases in preovulatory follicles, because of a decrease in low molecular weight IGF-binding proteins (IGFBPs), i.e. IGFBP2 and IGFBP4. In contrast, in ruminants, intrafollicular concentrations of these IGFBPs, as well as that of IGFBP5, dramatically increase in atretic follicles, leading to a strong decrease in IGF bioavailability. These changes in IGFBP levels are due to the changes in local expression as well as the changes in intrafollicular proteolytic degradation by pregnancy-associated plasma protein-A (PAPP; Mazerbourg *et al.* 2003).

Various *in vivo* mammalian models suggest a link between body size and IGF1 levels in serum, and several parameters of female reproduction such as fertility, ovulation rate and size of preovulatory follicles. The first evidence comes from mice knocked-out for the GH receptor (Bachelot *et al.* 2002), and transgenic female mice overexpressing IGFBP1 ubiquitously (particularly in the ovary (Huang *et al.* 1997)) or specifically in the liver (Froment *et al.* 2002). All strains present a low body weight, low IGF1 levels, and bioavailability, and a reduction in natural and induced ovulation rates. These reproductive abnormalities were associated with a dramatic reduction in the number of corpora lutea, and a lower number of healthy follicles > 200 µm, but not of those < 200 µm. Among the follicles > 200 µm, the ratio atretic:healthy was also increased in both transgenic mouse models, suggesting an alteration of terminal follicular development rather than basal follicular development (Bachelot *et al.* 2002, Froment *et al.* 2002). All these alterations are also illustrated in IGF1-null mice which also exhibit a marked reduction in body weight associated with sterility (Baker *et al.* 1996). As in the previous animal models, the ovarian follicular population does not appear to be affected up to the early antral stage, confirming

that in the mouse, IGF1 is not required for the recruitment of primordial follicles or for the growth of preantral follicles (Baker *et al.* 1996). In contrast, ovaries from IGF1 knockout mice (Baker *et al.* 1996) do not contain any antral follicles, and are thus unable to ovulate, even after a treatment with exogenous gonadotropins. Overall, these results strongly support the idea that IGF1 plays a key role in the responsiveness of the ovary to FSH during terminal follicular development, while it also plays a major role in the control of body weight.

The dog is one of the land vertebrate species exhibiting the widest span of body weight and height, from 500 g and 20 cm at withers (Chihuahua), to more than 80 kg and 100 cm at withers (Irish Wolfhound, Great Dane). In the dog as in other species, the height is known to be positively correlated with IGF1 serum concentrations (Eigenmann *et al.* 1984). The present work was designed to study the main parameters of ovarian physiology in a population of dogs exhibiting extreme differences in weight and height in order to evaluate the relationship between growth and female fertility. We measured IGF1, IGF2, and IGFBP3 concentrations in plasma and follicular fluid from preovulatory follicles, and studied the correlation between the assay values obtained and the number and size of preovulatory follicles, as well as the steroid concentrations in plasma and follicular fluid.

Materials and Methods

Unless otherwise indicated, all the chemicals used were purchased from Sigma–Aldrich.

Animals and monitoring of ovarian cycles

Forty bitches (2.5 ± 0.4 years; from 0.58 to 8.1 years old) were included in this study, 23 Beagle bitches from our experimental kennel and 17 bitches from private owners. These 17 bitches were either mongrels (2) or purebreed (Chihuahua, Yorkshire Terrier, Jack Russel Terrier, Bichon Maltais, English Bulldog, Brittany Spaniel, Braque Français, Belgian Malinois, Australian Shepherd, 2 Boxers, English Pointer, Dogo Argentino, Bernese Mountain Dog, and German Dogue). For each bitch, height at withers and weight were recorded.

Out of the 40 bitches used in this study, 22 bitches may be classified as ‘small’, i.e. <40 cm, 9 bitches were ‘medium’ (40–50 cm), and 9 bitches were ‘large’ (>50 cm; Supplementary Table 1, see section on supplementary data given at the end of this article). In the small and medium breeds, all the animals were adult, growth being completed around 8–12 months of age, but in the large breeds, the growth phase can last until 14–20 months. Among our nine ‘large’ bitches, five were adults, and four bitches (9–11 month old, 54–70 cm at withers, 18–38 kg) were probably not.

For the Beagle bitches, ovarian cycles were followed weekly by vaginal smears coupled with Harris–Shorr staining.

Heat initiation was considered when >80% of the cells were cornified (Johnston *et al.* 2001). For bitches from private owners, heat initiation was defined by the appearance of a vaginal bloody discharge. Thereafter, for all the bitches, follicular growth was followed by transabdominal ultrasonography with a curvilinear probe, 7.5 MHz (England & Yeager 1993, Reynaud *et al.* 2005) and progesterone blood levels (Concannon *et al.* 1989). Blood collection was performed at the cephalic or jugular vein. Plasma progesterone levels were assayed daily until ovariectomy (enhanced chemiluminescence Elecsys kit, Roche Diagnostics; intra-assay and inter-assay coefficients of variation <2%). When progesterone levels in plasma reached 0.5 ng/ml, blood was collected three times a day, and plasma samples were stored at -20°C until assayed, a posteriori, for LH concentrations (ELISA method; kit LH detect; INRA, Nouzilly, France; Guerin *et al.* 1997).

This protocol was approved by the Ethics Committee of the National Veterinary School of Alfort.

Follicular fluid collection

Ovariectomies were performed during follicular phase using a conventional surgical procedure (Fingland 1998). Ovarian bursa (with ovaries, oviducts and the tip of the uterine horns) was collected. Follicles present on the ovaries were counted and measured by gross examination under light, and all the follicles >2 mm were punctured individually with stretched Pasteur pipettes. The punctured liquids were observed under a stereomicroscope to collect the cumulus–oocyte complexes and observe mucification, and then follicular fluids, after centrifugation and supernatant collection, were immediately stored at -20°C . All the blood-contaminated fluids were discarded.

Steroid assays (progesterone and estradiol)

In follicular fluid and plasma samples collected on the day of neutering, progesterone and estradiol-17 β (E_2) were assayed by RIA. Follicular fluids were first diluted 1/1000, 1/5000, and 1/50 000 in phosphate buffer 0.1 M pH 7 (sodium azide 1 g/l, NaCl 0.155 M, Na_2HPO_4 0.072 M, NaH_2PO_4 0.028 M, and gelatin 1 g/l), and then assayed directly. Progesterone measurements were performed as previously described (Terqui & Thimonier 1974, Saumande 1991). E_2 was assayed with a kit [^{125}I] E_2 Diasorin (Stillwater, MN, USA), and results were compared with a standard curve. For measurements in plasma, steroids were extracted before assays.

IGF1 and IGF2 assays

Plasma or follicular fluid samples (25 μl) were incubated in 1 ml of 0.01 M HCl for 30 min at room temperature to dissociate IGFs from IGFBPs, then ultrafiltered on Amicon ultra-4 30 kDa (Millipore SAS, St Quentin-en-Yvelines, France) to separate IGFs from IGFBPs. The ultrafiltrate

containing IGFs was lyophilized, then taken up in a solution containing 0.03 M NaH₂PO₄, 500 µl/l Tween-20, 200 mg/l protamine sulfate, 200 mg/l NaNO₃, and 3.72 g/l EDTA (pH 7.4), and incubated for 2 days in a final volume of 500 µl with a specific polyclonal antihuman IGF1 antibody (1:120 000 dilution) that crossreacts with canine IGF1 (gift from J Closset, Centre Hospitalo-Universitaire de Liege, Belgium) and [¹²⁵I]-hIGF1 (10 000 c.p.m./tube). [¹²⁵I]-hIGF1 was obtained by iodination of hIGF1 using the iodogene method. For IGF2, samples were incubated with a monoclonal IGF2 antibody (1:1 000 000; Amano Pharmaceutical Co. Ltd, Nagoya, Japan), which crossreacts with canine IGF2 and [¹²⁵I]-hIGF2 (10 000 c.p.m./tube). Iodination was performed using the iodogene method. After incubation, free and bound IGFs were separated using albumin-coated charcoal. Samples were tested in triplicate plus two negative controls (without antibody). The threshold sensitivity of the assay was 0.5 ng/ml plasma. Depending on the assays, the intra-assay variation was between 4 and 5%, and inter-assay variation was 10%.

Western ligand blotting

Western ligand blotting (WLB) was performed as previously described (Monget *et al.* 1993). Samples (2.5 µl of plasma or follicular fluid) were submitted to electrophoresis on a 12% SDS-polyacrylamide gel under nonreducing conditions. In each gel, a standard canine follicular fluid was included. The proteins were then electrotransferred onto nitrocellulose filters (0.2-µm pore size) overnight at 4 °C. Filters were treated successively with PBS (0.01 M; pH 7.4) containing 0.1% Nonidet P-40, 0.5% gelatin, and 0.1% Tween-20, then incubated overnight at 4 °C with [¹²⁵I]-IGF2 in a solution containing 0.03 M NaH₂PO₄, 500 µl/l Tween-20, 200 mg/l protamine sulfate, 200 mg/l NaNO₃, and 3.72 g/l EDTA (pH 7.4). Afterwards, filters were washed with PBS containing 0.1% Tween-20, air-dried, and exposed to Hyperfilm MP (Amersham, GE Healthcare Life Sciences Europe GmbH, Orsay, France) with an intensifying screen at -70 °C or to a phosphor screen for quantification by a phosphoimager (Storm/Image Quant, Molecular Dynamics, Bondoufle, France).

Cleavage of IGFBP4 by follicular fluid

Characterization of IGFBP4 proteolytic degradation was performed on follicular fluid from preovulatory canine follicles as previously described (Mazerbourg *et al.* 1999). Briefly, 2.5 µl of canine follicular fluid were incubated in a solution of 20 mM Tris (pH 7.6) containing 137 mM NaCl (TBS) and 0.1% BSA with IGFBP4, with or without IGF1 or IGF2 for 20 h at 37 °C (final volume, 10 µl). In immunoneutralization experiments, an antibody raised against human PAPP-A, or a nonspecific rabbit IgG or glycerol as controls, was added to the incubation medium. At the end of the incubation time, the samples were analyzed by WLB.

Quantification of WLB

WLB was quantified by a phosphoimager (Storm/Image Quant, Molecular Dynamics) as previously described (Mazerbourg *et al.* 1999). The amount of radiolabeled IGF2 bound to IGFBP3 was expressed as the ratio of the integrated optical density (IOD) of the corresponding band, expressed in arbitrary units, to IOD of IGFBP3 from the standard follicular fluid loaded on each gel.

The quantification of IGFBP4 degradation by follicular fluid was measured by the difference $I_{-20} - I_{37}$, where I_{-20} is the IOD of the IGFBP4 band from samples not incubated, and I_{37} is the IOD of the IGFBP band from samples incubated at 37 °C.

Statistical analysis

All experimental data are expressed as the mean $\pm$ S.E.M. The degree of linear relationship between two variables was analyzed using Pearson's correlation coefficient. One-way ANOVA followed by the Tukey's or the Newmann-Keuls test was performed to test differences between means. Means of inhibition of IGFBP4 proteolytic degradation by antibody against PAPP-A were compared with 0 by a paired *t*-test. In the case of heterogeneity of variance, the Mann and Whitney test or the Kruskal-Wallis test was used to compare means between groups. Differences with $P < 0.05$ were considered significant.

Results

Animals and monitoring of ovarian cycles

The height at withers of the bitches ranged from 21 to 70 cm, and the weight at withers of the bitches ranged from 1.6 to 38.5 kg. Distribution of the heights and weights is presented in [Supplementary Figure 1](#), see section on [supplementary data](#) given at the end of this article. A linear correlation was observed between the two parameters ($r = 0.93$; $P < 0.0001$).

At the time of surgery, 15 bitches were at the pre-LH stage, i.e. they presented a proestrus/estrus vaginal smear, low levels of progesterone in plasma, and no detectable LH rise in plasma samples. Moreover, follicles larger than 2 mm were visible at ultrasonography. The plasma progesterone levels in those bitches were 1.20 ± 0.23 ng/ml (from 0.197 to 3.41). In total, 8, 4, and 3 bitches were at the pre-LH stage in the small, medium, and large group respectively.

Twenty-five bitches were at the post-LH/preovulatory stage, displaying an estrus vaginal smear, with progesterone in plasma at preovulatory levels (2–6 ng/ml), a LH peak and large antral follicles > 3 mm still visible by ultrasonography (ovulation had not occurred yet). These post-LH bitches had a plasma progesterone level at 4.66 ± 0.34 ng/ml (from 1.82 to 8.57). In total, 14, 5, and 6 bitches were at the post-LH stage in the small, medium, and large group respectively.

Figure 1 Correlation between IGF1 concentrations (ng/ml) in canine plasma and follicular fluid. Twenty-five microliters of canine plasma or follicular fluid (follicles ≥ 4.5 mm) were assayed for IGF1 as described in Materials and Methods. Assays were performed on 105 follicular fluids from 35 bitches. Depending on the animal, the IGF1 concentration represents the mean of 1–7 preovulatory follicles. $r=0.85$, $P<0.001$.

Before the LH peak, follicles considered as preovulatory were large (≥ 4.5 mm) and presented high E_2 levels (>250 ng/ml) in their follicular fluid. After the LH peak, follicles considered as preovulatory were large (≥ 4.5 mm) and contained an oocyte with a fully mucified cumulus mass.

Follicular fluids

From the 40 bitches in this study, a total of 455 follicles were measured and punctured (13.5 ± 0.7 follicles per bitch). They ranged from 2 to 8 mm in diameter. Out of these follicles, 249 (54.7%) follicular fluids were recovered and stored at -20°C for further analysis. The other follicles were either too small, i.e. <3 mm, to collect follicular fluid (102 follicles, 22.4%), or the follicular fluid samples obtained by puncture were contaminated with blood (104 follicles, 22.9%).

From the 15 bitches neutered at the pre-LH stage, a total of 203 follicles ranging from 2 to 7.5 mm were collected, with 96 follicles of 2–4.5-mm diameter and 107 follicles of 4.5–7.5-mm diameter. Only follicles ≥ 4.5 mm whose follicular fluid contained more than 250 ng/ml E_2 were considered as preovulatory. Follicles that were 2–4 mm in diameter were considered as atretic (Reynaud *et al.* 2009).

At the post-LH/preovulatory stage (20 bitches), a total of 252 follicles ranging from 2 to 8 mm were punctured, with 61 follicles between 2 and 4 mm and 191 follicles between 4.5 and 8 mm. Only follicles ≥ 4.5 mm were considered as preovulatory. All contained a mucified cumulus–oocyte complex.

IGF1, IGF2, and IGFBP3 levels in plasma and follicular fluids

Only follicles with at least 30 μl of follicular fluid available have been assayed for IGF1, IGF2, and IGF-BP. Within the

same breed (10 Beagle bitches at the pre-LH stage and 12 Beagle bitches at the post-LH stage), levels of IGF1 in plasma and follicular fluid samples collected before and after LH did not demonstrate any significant differences: 61.3 ng/ml in plasma and 46.0 ng/ml in follicular fluid before LH versus 57.0 ng/ml in plasma and 47.3 ng/ml in follicular fluid after LH (Supplementary Table 2, see section on supplementary data given at the end of this article). No difference was observed either for IGF2 (Supplementary Table 2). This lack of difference within a same breed (so without interaction with other breeds) led us to pool the results from pre- and post-LH bitches for the study of the IGF system.

RIAs were performed on 105 follicles (1–7 follicles per bitch, 4.5–8-mm follicles) and 35 plasma samples. No significant correlation was observed between intrafollicular IGF1 concentration and follicle diameter ($r=0.03$). Within a given bitch, intrafollicular levels of IGF1 were positively correlated with plasma levels ($r=0.85$, $P<0.001$, Fig. 1). Furthermore, the plasma IGF1 levels were positively correlated with both the height ($r=0.45$, $P<0.01$) and weight ($r=0.55$, $P<0.01$) of bitches. The concentrations were threefold higher in large dogs than in small dogs ($r=0.45$, $P<0.01$, Fig. 2).

Analysis of IGF2 (assayed in 107 follicles from 4.5 to 8 mm and in 38 plasma samples) revealed no significant relationship between intrafollicular IGF2 concentration and follicle diameter ($r=0.08$). Intrafollicular levels of IGF2 were found to be positively correlated with corresponding plasma levels ($r=0.76$, $P<0.001$, Supplementary Figure 2, see section on supplementary data given at the end of this article). Intrafollicular IGF2 was also positively correlated with intrafollicular IGF1 concentrations ($r=0.49$, $P<0.01$). However, no significant correlation was observed between IGF2 levels and bitch height ($r=0.08$).

Figure 2 Effects of bitch height class on the concentrations of IGF1 in plasma (open bar) and follicular fluid (solid bar). Three size classes were analyzed: bitches <40 cm ($n=22$), 40–50 cm ($n=9$), and >50 cm ($n=9$). Results are expressed as the mean $\pm$ S.E.M. Letters above the bars indicate that these groups differ significantly from each other (a versus b: $P<0.01$).

Figure 3 Western ligand blotting for detection of IGFBPs in follicular fluids and plasmas. Samples (2.5 µl) were submitted to western ligand blotting as described in Materials and Methods. (A) IGFBPs in follicular fluids and plasmas from a large (bitch 1), a medium (bitch 2), and a small (bitch 3) bitch. (B) IGFBP in follicular fluids and plasmas from three other large (bitch 4), medium (bitch 5), and small (bitch 6) animals. FCS and standard follicular fluid were loaded on the gel as controls to standardize quantification.

WLB was carried out on 44 follicular fluid samples and on plasma samples from 23 bitches (Fig. 3A and B). Quantification of WLB showed a positive correlation ($r=0.78$, $P<0.001$) between intrafollicular and plasmatic IGFBP3 levels. Plasmatic IGFBP3 levels were also positively correlated with both height and weight of bitches ($r=0.59$, $P<0.02$, [Supplementary Figure 3](#), see section on [supplementary data](#) given at the end of this article). Moreover, a correlation was found between IGF1 and IGFBP3 levels in plasma ($r=0.79$, $P<0.001$, [Supplementary Figure 4](#), see section on [supplementary data](#) given at the end of this article). By contrast, no correlation between IGF2 and IGFBP3 levels was observed ($r=0.06$, $P>0.05$).

Moreover, follicular fluid of canine preovulatory follicles was able to degrade IGFBP4, this degradation being neutralized by the addition of an antibody raised against human PAPPA ([Supplementary Table 3](#), see section on [supplementary data](#) given at the end of this article).

Characterization of follicle population in the canine preovulatory ovary with regard to bitch height

Interestingly, the number of preovulatory follicles was significantly and positively correlated with bitch height at both the pre-LH ($n=15$ bitches, $r=0.56$, $P<0.05$) and the post-LH ($n=25$ bitches, $r=0.63$, $P<0.001$) stages (Fig. 4).

Moreover, considering the three largest preovulatory follicles in each bitch, the mean diameter of these three follicles was shown to be positively correlated with bitch height, at both stages (pre-LH: $r=0.70$, $P<0.01$ and post-LH: $r=0.66$, $P<0.001$, Fig. 5). Of note, the mean diameter of preovulatory follicles was highly homogeneous within each bitch (data not shown). No interaction between progesterone concentration (and, as a consequence, time interval before

and after LH) and the mean diameter of the three largest follicles was observed.

The age effect (from 0.58 to 8.1 years old) was analyzed in Beagle bitches, and no significant effect of aging was observed on IGF1 concentrations in follicular fluid, on numbers of >4.5 -mm follicles and on the diameter of the three largest follicles.

Concentrations of steroids (E_2 and progesterone) in plasma and follicular fluid

E_2 concentrations were determined in plasma from 36 bitches ($n=15$ at the pre-LH stage and $n=21$ at the post-LH stage) and in 158 follicular fluids (61 fluids collected from 4.5 to 7.5-mm follicles at pre-LH stage and 97 fluids collected from 4.5 to 8-mm follicles at the post-LH stage). Levels of E_2 in plasma before and after LH did not demonstrate any significant differences ([Supplementary Table 4](#), see section on [supplementary data](#) given at the end of this article). Plasmatic and intrafollicular levels of E_2 were not correlated ($P=0.08$).

On the contrary, E_2 levels in follicular fluid were significantly higher at the pre-LH stage than at the post-LH stage ($P<0.001$). Before LH ($n=15$ bitches, 61 follicles), mean intrafollicular E_2 concentration was 824.9 ± 52.5 ng/ml (267–1590 ng/ml) vs 43.1 ± 9.0 ng/ml in follicles after LH (4–161 ng/ml, $n=21$ bitches, 97 follicles). Levels of E_2 in follicular fluid were very high (15 000 times higher than in plasma at the pre-LH stage). Follicular E_2 levels and bitch heights were negatively correlated ($r=-0.58$, $P<0.05$, Fig. 6).

Progesterone was assayed in plasma from 38 bitches ($n=15$ at the pre-LH stage and $n=22$ at the post-LH stage) and in 168 follicular fluids (71 samples collected from 4.5 to 7.5-mm follicles at pre-LH stage and 96 samples collected from 4.5 to 8-mm follicles at the post-LH stage; [Supplementary Table 4](#)).

Figure 4 Number of large follicles ≥ 4.5 mm in pre-LH (white circles and grey regression line) and post-LH (black rhombs and black regression line) canine ovaries, in relation to bitch height. After ovariectomies, all the follicles ≥ 4.5 mm were measured and counted in 15 bitches at the pre-LH stage and 25 bitches at the post-LH stage. Pre-LH: $r=0.56$, $P<0.05$ and post-LH: $r=0.63$, $P<0.001$.

Figure 5 Mean diameter of the three largest follicles in pre-LH (white circles and grey regression line; $n=15$ bitches, 45 follicles) and post-LH (black rhombs and black regression line, $n=25$ bitches, 75 follicles) canine ovaries, in relation to bitch height at withers. Pre-LH: $r=0.70$, $P<0.01$ and post-LH: $r=0.66$, $P<0.001$.

In both plasma and follicular fluids, levels were significantly higher at the post-LH stage than at the pre-LH stages ($P<0.001$). Like with E_2 , intrafollicular concentrations of progesterone were very high and 1200–1300 times higher than in plasma. At both stages, no effect of follicle diameter on intrafollicular progesterone was observed ($r=0.08$ at the pre-LH stage and $r=0.30$ at the post-LH stage). No correlation between plasmatic/follicular progesterone at pre/post-LH and bitch height was observed.

Intrafollicular progesterone was positively correlated with both plasmatic ($r=0.57$, $P<0.05$) and intrafollicular IGF1 at the pre-LH stage ($r=0.68$, $P<0.05$). At the pre-LH stage, plasmatic progesterone and intrafollicular IGF2 were positively correlated ($r=0.75$, $P<0.01$).

Discussion

Our results suggest that the wide span in body height among dogs from different breeds is associated with dramatic differences in IGF1, as well as in IGFBP3 levels, in both plasma and follicular fluid from preovulatory follicles. These differences are associated with significant differences in some characteristics of ovarian folliculogenesis. In particular, large dogs have a higher number of preovulatory follicles than small dogs, these follicles being 70% larger in the largest (70 cm) dog than in the smallest (21 cm) dog. However, concentrations of E_2 in follicular fluid from preovulatory follicles (but not plasma) were lower in large dogs.

A relationship between plasmatic IGF1 concentrations and dog size had been described previously (Eigenmann *et al.* 1984) but no data were available on follicular fluids, probably due to the difficulty of collecting follicle fluid. Ovulation in the dog occurs only twice a year, with no current means of pharmacological control. Moreover, follicles reach a size allowing puncture for only a few days before ovulation, which

requires a thorough clinical follow-up (England *et al.* 2008, Reynaud *et al.* 2009).

The positive correlation between IGF1 and IGFBP3 plasma levels, and body weight of dogs had been previously reported by Maxwell *et al.* (1998). However, the bands corresponding to IGFBP3 in WLB in his study were not quantified. Also, we did not find differences in plasma IGF2 levels between small and large dogs, like previously described by Maxwell *et al.* (1998). In this study, we measured body height rather than body weight of animals, since weight depends not only on height but also on body mass index. Interestingly, in the canine species, an SNP has been recently found to be a major determinant of small size in *IGF1* gene (Sutter *et al.* 2007). Overall, this is in keeping with results obtained in several other species (Mazerbourg *et al.* 2003) showing that IGF1 and IGFBP3 plasma concentrations are closely associated with body size.

Moreover, the positive correlation found between IGF1 and IGFBP3 levels is supported by the fact that in plasma, most of IGF1 is bound to IGFBP3, in a 150-kDa complex, and that the synthesis of IGFBP3 in the liver is stimulated by IGF1 (Bale & Conover 1992) and GH.

Interestingly, we found a positive correlation between plasmatic and intrafollicular levels (preovulatory follicles) of three components of the IGF system: IGF1, IGF2, and IGFBP3. This had previously been shown in women, mares, cows, and ewes (Mazerbourg *et al.* 2003), which strengthens arguments in favor of a major seric origin for IGF1 in large antral follicles. First, in several species, as stated, IGF1 concentrations in large normal follicles are slightly lower and positively correlated with serum levels (sheep (Monget *et al.* 1993)). Secondly, the systemic 150-kDa IGFBP-IGF complex, which is secreted by the liver and carries more than

Figure 6 Relationship between follicular concentrations of estradiol-17 β at the pre-LH stage (61 follicles from 14 bitches) and the bitch height. After collection, estradiol-17 β levels were assayed by RIA. Only preovulatory follicles, measuring >4.5 mm and presenting high estradiol levels (>250 ng/ml) in their follicular fluid were considered. Preovulatory follicles from small bitches contained higher concentrations of estradiol than preovulatory follicles from large bitches ($r=-0.58$, $P<0.05$).

90% of IGFs of liver origin in the serum, has been identified in ovine (Hodgkinson *et al.* 1989) and human follicular fluid (Hughes *et al.* 1997), proteins with molecular weights below 500 kDa being able to cross the basal membrane of the follicle (Shalgi *et al.* 1973). Thirdly, immunization of cattle against the GH-releasing factor leads to a decrease in IGF1 levels in both serum and follicular fluid of large follicles (Stanko *et al.* 1994a). In contrast, treatment of cattle with GH leads to an increase in IGF1 levels in both compartments (Stanko *et al.* 1994b). Hence, every change in IGF1 levels in serum seems to have a direct impact on the level in large antral follicles.

In this study, an increase in the number and the diameter of preovulatory follicles in large bitches compared with small bitches was observed. These observations are in agreement with the data describing higher ovulation rates in canine larger breeds (10.1 vs 5.5 ovulations (Reynaud *et al.* 2006)) and with the mean higher litter size recorded in large breeds: 6.9 puppies in the Irish Wolfhound breed (100 cm at withers) versus 2.8 puppies in the Chihuahua breed (20 cm at withers) (Leroy 2007). The increase in the number of preovulatory follicles may be due to the huge differences in plasma and intrafollicular IGF concentrations between larger and smaller animals. Indeed, IGF1 is known to stimulate follicular growth in several species, and flushing treatments, as well as insulin injections are able to increase both IGF1 levels in serum, and the size and the number of ovulatory follicles (for review Monget & Martin (1997) and Silva *et al.* (2009)). Moreover, in several models of IGF1-deficient mice, a decrease in the number of antral FSH-dependent follicles due to an increase in the rate of atresia has been reported (Bachelot *et al.* 2002, Froment *et al.* 2002). In bovine, a mutation on the GH gene in Brahman cattle (McCormack *et al.* 2009) leads to a 30% decrease in adult body weight, a seven- to eightfold decrease in IGF1 levels, a two- to fourfold decrease in the number of 2–5-mm diameter follicles, and a decrease in the number of large (> 5 mm diameter) follicles without any change in FSH, LH, and E₂ levels (Chase *et al.* 1998). Furthermore, FSH, insulin, GH, and IGF1 may interact to regulate follicle numbers, as described in Angus, Brahman, and Senepol cows (Alvarez *et al.* 2000). So, in cattle like in the mouse, IGF1 seems to play a key role in increasing the sensitivity to gonadotropin of small antral follicles (200 µm in the mouse and 5 mm diameter in cattle), and plays a key role in terminal follicular growth. So, it appears likely that IGF1 plays partly a similar role in large dogs, and increases the number and the diameter of preovulatory follicles.

The bone morphogenetic protein (BMP) family also plays a key role in regulating number of ovulatory follicles and their size. Thus, ewes that carry a partial loss-of-function of the BMPR-IB or BMP-15 gene exhibit an increase in ovulation rate as well as a decrease in ovulatory follicle diameter (for review McNatty *et al.* (2005)). So, it may be that some differences exist between dog breeds of different sizes in BMP system functionality.

Intrafollicular concentrations of E₂ and progesterone in bitches have been reported once (Metcalf 1999) but only at

the preovulatory stage and without data on the effects of follicle size. In our study, the mean concentrations of progesterone (6500–24 800 ng/ml) and E₂ (4–161 ng/ml) were in accord with previously reported levels (3900–12 800 ng/ml and 2–52 ng/ml for progesterone and E₂ respectively (Metcalf 1999)).

The higher intrafollicular level of E₂ in large preovulatory follicles of small in comparison with large dogs may be due to their smaller diameter, leading to a higher concentration. Moreover, the absence of difference in plasma E₂ levels in the various dog breeds suggests that total intrafollicular and plasma levels of IGF1 and IGF2 may not be a pertinent parameter to investigate the level of differentiation of large antral follicles. IGF bioavailability is likely a more relevant criterion. In particular, we observed in this study that follicular fluid from preovulatory follicles contains PAPP-A able to degrade IGFBP4, as previously described in humans, cattle, horses, pigs, and sheep (Mazerbourg *et al.* 2003). This suggests that in dogs like in other mammals, IGF bioavailability is maximal in large preovulatory follicles, leading to an optimal sensitization of granulosa cells to FSH. It is likely that free IGF, in contrast to total IGF concentrations, is similar in preovulatory follicles from the different dog breeds studied. Moreover, our present work strongly suggests that among all the elements of the IGF system, PAPP-A (as well as IGFBP2, IGFBP4 and, in ruminants, IGFBP5) rather than IGF1 and IGFBP3, is the true paracrine factor, while the latter are endocrine factors.

In conclusion, the present work demonstrates that in dogs, IGF1 and IGFBP3 concentrations in plasma and preovulatory follicles are highly correlated with body height, which in turn is correlated with the number and the size of ovulatory follicles. The precise role of the IGF system in this control of ovarian folliculogenesis and its interaction with gonadotropins remain to be investigated.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/JOE-09-0450>.

Declaration of interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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