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Validation of a noninvasive diagnostic tool to verify neuter status in dogs: The urinary FSH to creatinine ratio

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ABSTRACT

Determining the presence of functional gonadal tissue in dogs can be challenging, especially in bitches during anestrus or not known to have been ovariectomized, or in male dogs with nonscrotal testes. Furthermore, in male dogs treated with deslorelin, a slow-release GnRH agonist implant for reversible chemical castration, the verification of complete downregulation of the hypothalamic-pituitary-gonadal (HPG) axis can be difficult, especially if pretreatment parameters such as the size of the testes or prostate gland are not available. The aims of this study were to validate an immunoradiometric assay for measurement of FSH in canine urine, to determine if the urinary FSH to creatinine ratio can be used to verify the neuter status in bitches and male dogs, as an alternative to the plasma FSH concentration, and to determine if downregulation of the HPG axis is achieved in male dogs during deslorelin treatment. Recovery of added canine FSH and serial dilutions of urine reported that the immunoradiometric assay measures urinary FSH concentration accurately and with high precision. Plasma FSH concentrations (the mean of two samples, taken 40 minutes apart) and the urinary FSH to creatinine ratio were determined before gonadectomy and 140 days (median, range 121–225 days) and 206 days (median, range 158–294 days) after gonadectomy of 13 bitches and five male dogs, respectively, and in 13 male dogs before and 132 days (median, range 117–174 days) after administration of a deslorelin implant. In both bitches and male dogs, the plasma FSH concentration and the urinary FSH to creatinine ratio were significantly higher after gonadectomy, with no overlapping of their ranges. Receiver operating characteristic analysis of the urinary FSH to creatinine ratio revealed a cut-off value of 2.9 in bitches and 6.5 in males to verify the presence or absence of functional gonadal tissue. In male dogs treated with deslorelin, the plasma FSH concentrations and urinary FSH to creatinine ratios were significantly lower after administration of the implant, but their ranges overlapped. We conclude that the urinary FSH to creatinine ratio can be used to verify the neuter status of bitches and male dogs. However, it cannot be used for the assessment of complete downregulation of the HPG axis after administration of a deslorelin implant. The urinary FSH to creatinine ratio is preferable over the plasma FSH concentration because it involves only one sample that can be collected relatively easy and noninvasively.

1. Introduction

The bitch has a nonseasonal, monestrous cycle characterized by a follicular phase with spontaneous ovulations, followed by a luteal phase of about 2 months, almost

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irrespective of whether the bitch is pregnant, and a nonseasonal anestrus of 2 to 10 months [1]. As in other species, the endocrine control of reproduction is exerted by the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotropic releasing hormone (GnRH) is secreted by the hypothalamus and stimulates the gonadotropes of the pituitary gland to secrete LH and FSH. These stimulate the gonads to produce sex steroids, which exert negative and/or positive feedback on the hypothalamus through interaction with kisspeptin neurons. Kisspeptin directly stimulates GnRH neurons to secrete GnRH [2,3]. The secretion of GnRH is pulsatile and thus secretion of LH and FSH is also pulsatile, as is that of all hormones secreted by the adenohypophysis [4,5].

When it is unknown whether an ovariectomy has been performed, the presence of functional gonadal tissue in bitches can easily be verified by vaginoscopy and/or vaginal cytology during proestrus and estrus, revealing changes induced by high-circulating levels of estradiol, and by measuring plasma progesterone concentration during the luteal phase. However, it may be difficult to distinguish ovariectomized from intact bitches during anestrus [6]. Abdominal ultrasound may be used to determine if ovaries are present or not. However, the sensitivity and specificity of this technique depends on the level of experience of the sonographer and the specifications of the equipment used, for example, the transducer [7]. During the follicular phase and luteal phase, it is more likely that the ovaries can be identified by using abdominal ultrasound because of the presence of follicles and corpora lutea respectively. In contrast, during anestrus, the ovaries are smaller and have a more homogenous appearance (absence of large follicles and corpora lutea) and are therefore more challenging to identify, especially in large dogs or in dogs with a large amount of abdominal fat tissue [7,8]. In addition, Buijtels et al.[9] described two populations of dogs: bitches with remnant ovarian tissue and dogs with a disorder of sexual development [10]. In both studies, abdominal ultrasound was used to verify the presence and localization of gonadal tissue in addition to a provocative GnRH stimulation test, and thereafter laparotomy and histology of the surgically removed tissue was performed. In both studies, it appeared that the results of the abdominal ultrasound were not always conclusive. Therefore, the use of abdominal ultrasound alone cannot be used to either confirm or rule out the presence of functional ovarian tissue.

In both males and females, mean basal plasma LH and FSH concentrations increase after gonadectomy, due to loss of negative feedback of gonadal hormones [11–13]. Basal plasma FSH concentration is higher in ovariectomized bitches than in intact anestrous bitches, and the ranges do not overlap [12,13]. In contrast, the ranges of basal plasma LH and estradiol concentration in ovariectomized bitches and intact anestrous bitches do overlap [12,13]. On the basis of these findings, measurement of plasma FSH concentration appears to be the most reliable means of verifying the presence of ovarian tissue during anestrus. However, a single measurement of plasma FSH concentration may not be reliable for this purpose because gonadotropin secretion is pulsatile. Kooistra et al.[4] (1999) have reported that there are one to two LH and FSH pulses per 6 hours during the luteal phase and anestrus. Hence, multiple blood samples are required to circumvent the effect of pulsatile secretion on the interpretation of gonadotropin concentrations.

Another means of verifying the neuter status of dogs is the measurement of plasma anti-mullerian-hormone (AMH) concentration. The ovaries are considered to be the sole source of AMH, and therefore, it can be expected that AMH will be a specific indicator of the presence of functional gonadal tissue. Plasma AMH concentration has been shown to be higher in intact dogs than in ovariectomized dogs but with some overlapping of the ranges [14]. Because of the overlapping and the large individual differences in plasma gonadotropin, estradiol, and AMH concentrations, the reliability of single measurement of the plasma concentration of one of these hormones during anestrus may not be sufficient to verify neuter status.

Provocative tests with exogenous GnRH can also be used to detect the presence of functional ovarian tissue. After intravenous injection of a GnRH analog, a significant rise in plasma estradiol concentration is observed in anestrous bitches but not in ovariectomized bitches [6]. However, the GnRH stimulation test has the disadvantages of being invasive, more than one blood sample is needed, and somewhat time consuming (maximum plasma estradiol concentration after GnRH injection is only reached after 60–120 minutes) [6,13].

Measurement of the FSH concentration in urine may be a useful alternative to determine the presence of functional ovarian tissue during anestrus. It reflects the plasma FSH concentration over a period of several hours and therefore is expected to compensate for pulsatile FSH secretion. To correct for changes in concentration of the urine, which influences the urinary FSH concentration, the urinary FSH to creatinine ratio should be used, in analogy to the previously validated corticoid to creatinine ratio [15]. Compared with other methods to verify the presence of functional gonadal tissue, for example a GnRH stimulation test, the collection of urine for measurement of the FSH concentration is easy and noninvasive.

Basal plasma testosterone concentration is much higher in intact male dogs than in gonadectomized males, and the ranges do not overlap [13]. Consequently, a single measurement of plasma testosterone concentration is a reliable means of verifying the presence of functional testicular tissue as, for example, when undescended testes are suspected and the dogs' history is unknown. It is more difficult to determine the functionality of the testicles during temporary chemical castration by the use of a slow-release GnRH agonist (deslorelin) implant. The GnRH receptor on the gonadotropes becomes desensitized after long-term stimulation [16]. The duration of downregulation of the HPG axis is dependent on the dose of deslorelin, albeit with large interindividual variation. The time to complete recovery of the HPG axis has been reported to vary between 360 and 680 days after implantation of 6-mg deslorelin [17]. Downregulation of the GnRH receptors on the gonadotropes results in a decrease in the plasma LH concentration [18]; effects on plasma FSH concentration have not been reported. To evaluate the function of the testicular tissue in a male dog treated with a

slow-release GnRH agonist, the size of the testicles and/or the prostate gland can be measured, as both will decrease in size when downregulation of the HPG axis is achieved [19,20]. However, the size of the testicles and/or the prostate will only be a reliable parameter if they can be compared with pretreatment size. If this is unknown, it can be difficult to ascertain that the deslorelin implant was administered correctly and that full downregulation of the HPG axis has been achieved. A noninvasive endocrine test to assess the function of the HPG axis would therefore be of great value for use in male dogs. Consequently, measurement of the FSH concentration in urine may be of interest in both bitches and male dogs.

The first aim of the present study was to validate a method for measurement of FSH in canine urine. The second aim was to determine the reliability of the plasma FSH concentration and the urinary FSH to creatinine ratio for verification of the presence of functional gonadal tissue in bitches and male dogs, and to assess if complete downregulation of the HPG axis is achieved in male dogs during deslorelin treatment.

2. Materials and methods

2.1. Animals and collection of blood and urine samples

13 healthy, client-owned bitches were used in this study. They were presented at our clinic for routine bilateral ovariectomy. According to their owners, all had been in estrus at least once and they were in anestrus at the time of inclusion in the study. Their median plasma progesterone concentration was 1.2 nmol/L (range 0.5–2.6 nmol/L). The group consisted of one American Bulldog, one Border Collie, one Bouvier des Flandres, one Bull Terrier, one Chow Chow, one Dachshund, one Labrador Retriever, one Siberian Husky, and five mixed-breed dogs. Their median age at the onset of the study was 18 months (range 10–73 months).

In addition, 18 healthy, client-owned male dogs were used. All had two scrotal testes. Five of these dogs were presented for routine bilateral orchiectomy and 13 for chemical castration with an implant containing 4.7-mg deslorelin (Suprelorin, Virbac Nederland BV, Barneveld, The Netherlands). The group in which bilateral orchiectomy was performed consisted of one Beagle, one Golden Retriever, one Saint Bernard, one Standard Poodle, and one Weimaraner. Their median age at the onset of the study was 22 months (range 20–48 months). The group of 13 male dogs that received a deslorelin implant consisted of one Bichon Friché, one Bouvier des Flandres, one Dutch Shepherd dog, one Flatcoated Retriever, one German Longhaired Pointer, two Labrador Retrievers, one Malinois, one Rottweiler, one Tibetan Terrier, 1 Västgötaspets, one White Shepherd dog, and one mixed-breed dog. Their median age at the onset of the study was 32 months (range 18–107 months).

Blood and urine samples were collected from each dog before and after gonadectomy or deslorelin treatment. Their owners were requested to collect a spontaneous morning urine sample from the dog on the day of its presentation to the clinic. On the same day, before gonadectomy or deslorelin treatment, two blood samples, 40 minutes apart, were collected from the jugular vein in heparinized tubes for measurement of the basal plasma FSH concentration. The mean concentration in these two samples was used for further analysis. In addition, two blood samples, taken 40 minutes apart, and one spontaneous urine sample were collected approximately one-half year after gonadectomy or deslorelin treatment (females: median 140 days, range 121–225 days; males: orchiectomy group: median 206 days, range 158–294 days; deslorelin group: median 132 days, range 117–174 days).

Plasma and urine for FSH determination were stored at −20 °C and −80 °C, respectively, until assayed.

2.2. Hormone measurements in plasma

Plasma FSH concentration was measured by an immunoradiometric assay (IRMA; AHROO4, IDS, Liège, Belgium), according to the manufacturer's instructions and as described previously [12]. The intraassay and interassay coefficients of variation (CVs) were 3.0% and 6.0%, respectively. The lower limit of quantitation was 0.5 μg/L.

Plasma progesterone concentration was measured by a previously validated I125-RIA [21]. The intraassay and interassay CVs were 6% and 10.8%, respectively. The lower limit of quantitation was 0.15 nmol/L.

2.3. Validation of the assay for measuring urinary FSH

Urinary FSH concentration was measured by the same IRMA used to measure plasma FSH concentration (AHROO4, IDS, Liège, Belgium). To determine its accuracy and precision, dog urine with an FSH concentration of 0-μg/L FSH (urine from a dog after treatment with a deslorelin implant) was used and highly purified canine FSH, kindly provided by the manufacturer (Biocode, Liège, Belgium), was added to provide a concentration of 127.8 μg/L. Stepwise dilutions with the FSH-free urine resulted in concentrations of 1:2 to 1:128. Each diluted sample was assayed five times. The manufacturer's standard curve ranged from 2.8 to 200 μg/L.

To correct for changes in urine concentration, which influences the urinary FSH concentration, the urine FSH to creatinine ratio was determined. Urinary creatinine was measured by the Jaffé alkaline picrate method (DXC 600 Unicel, Beckman Coulter, UVDL Diagnostic Laboratory, Utrecht, The Netherlands). The FSH to creatinine ratio was calculated by dividing the urine FSH concentration (in μg/L × 10^5) by the urine creatinine concentration (in μmol/L).

To determine the effect of storage conditions and duration on the urinary FSH concentration, a pilot study was performed by using the urine of four intact and three castrated dogs. The urine samples were divided into 24 vials and stored at room temperature, 4 °C, −20 °C, and −80 °C. The urinary FSH to creatinine ratio was determined at the day of collection of the urine and after 1, 3, 5, 7, and 9 weeks of storage at the different storage temperatures. The urinary FSH to creatinine ratio declined over time in most samples, compared with the first sample. The greatest decline was observed in urine stored at room temperature. There was individual variation in the influence of storage

temperature and duration of storage with the largest variation in urine stored at −20 °C. The least variability was observed in urine that was stored at −80 °C. Therefore, the urine samples used for the present study were stored at −80 °C.

2.4. Data analysis

Statistical analysis was performed using SPSS for Windows, version 22 (SPSS Inc., Chicago, USA).

Nonparametric tests were used because not all data were normally distributed. The plasma and urinary FSH concentrations before and after gonadectomy or chemical castration were analyzed by Wilcoxon signed-rank test.

Pearson's bivariate correlation test was performed to determine the correlation between plasma and urinary FSH concentrations before and after gonadectomy or deslorelin treatment. The Mann-Whitney test was used to compare the basal plasma FSH concentrations in intact bitches and male dogs.

In all analyses, $P < 0.05$ was considered significant.

If data range before and after gonadectomy or during deslorelin treatment did not overlap, cut-off values were calculated by receiver operating characteristic (ROC) analysis with gonadectomy and deslorelin treatment as positive states. Only cut-off values with 100% sensitivity and 100% specificity are given.

2.5. Ethics of experimentation

This study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Utrecht University, conform Dutch legislation. All owners of the dogs included in this study read and signed an informed consent form in which all procedures concerning this study were explained.

3. Results

3.1. Validation of IRMA for urinary FSH

Table 1 shows the results of measurements of FSH in the dilution series. The intraassay and interassay CVs were 2.7% and 4.3%, respectively. The lower limit of quantitation was 0.5 μg/L.

3.2. Plasma FSH concentration

The median plasma FSH concentration in bitches before gonadectomy was 4.4 μg/L (range 1.2–8.1 μg/L), which was lower ($P < 0.01$) than after gonadectomy (56.1 μg/L; range 17.5–82.9 μg/L).

The median plasma FSH concentration in male dogs before gonadectomy was 2.0 μg/L (range < 0.5–4.4 μg/L), which was lower ($P = 0.04$) than after gonadectomy (25.4 μg/L; range 8.9–59.7 μg/L).

The median plasma FSH concentration in male dogs before deslorelin treatment was 2.4 μg/L (range 0.9–6.9 μg/L), which was higher ($P < 0.01$) than during the treatment (<0.5 μg/L; range < 0.5–2.0 μg/L).

The median plasma FSH concentration in all male dogs ($n = 18$) before gonadectomy or deslorelin treatment was 2.2 μg/L (range < 0.5–6.9 μg/L), which was lower ($P = 0.02$) than in the bitches ($n = 13$). The difference in median plasma FSH concentration after gonadectomy between bitches ($n = 13$) and male dogs ($n = 5$) was not significant ($P = 0.05$).

Table 1
Accuracy and precision of the measurement of the FSH concentration in urine by IRMA.

Dilution	Added dose (μg/L)	Measured (μg/L)	SD (μg/L)	CV (%)[a]	Recovery (%)[b]
Undiluted	127.8	127.8	2.2	1.7	100
1:2	63.9	54.2	0.5	0.8	84.8
1:4	32.0	27.7	0.3	1.1	86.6
1:8	16.0	15.6	0.3	2.0	97.5
1:16	8.0	8.5	0.2	1.9	106.3
1:32	4.0	4.5	0.1	2.2	112.5
1:64	2.0	2.1	0.1	2.4	105.0
1:128	1.0	1.0	0.0	3.1	100

Highly purified canine FSH was added to canine urine to a concentration of 127.8 μg/L. Seven dilutions were then prepared, ranging from 1:2 to 1:128. The resulting concentrations of FSH were both calculated (added dose) and measured by IRMA (measured). Each dilution was assayed 5 times and the table shows the mean of five measurements for each.
Abbreviations: IRMA, immunoradiometric assay; SD, standard deviation.
[a] CV: coefficient of variation, which indicates the precision of the assay.
[b] Recovery is defined as the measured FSH concentration relative to the calculated added dose. The recovery indicates the accuracy of the assay.

3.3. The urinary FSH to creatinine ratio

The median urinary FSH to creatinine ratio in bitches before gonadectomy was 0.3 (range 0–2.6), which was lower ($P < 0.01$) than after gonadectomy (15.4; range 3.2–31; Fig. 1). Receiver operating characteristic analysis revealed a cut-off value of 2.9 for the urinary FSH

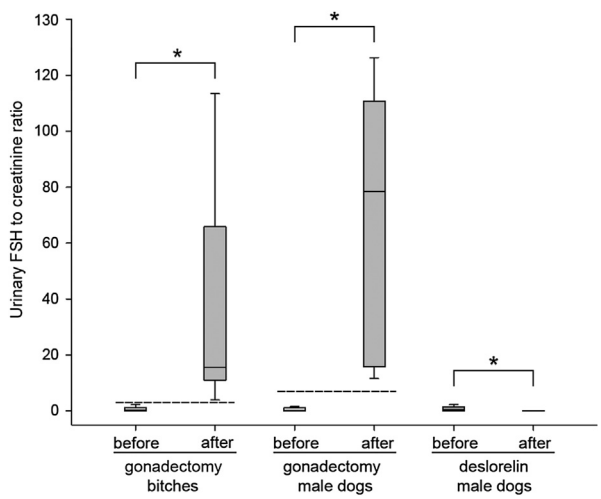

Fig. 1. The urinary FSH to creatinine ratios before and after gonadectomy in respectively 13 bitches (left) and five male dogs (middle), and in13 male dogs before and after the administration of deslorelin, a slow-release GnRH agonist implant (right). Asterisks denote a significant difference: *$P < 0.05$. The interrupted line reflects the cut-off values of 2.9 (females) and 6.5 (males).

to creatinine ratio to verify the presence or absence of functional ovarian tissue.

The median urinary FSH to creatinine ratio in male dogs before gonadectomy was 0 (range 0–1.6), which was lower ($P = 0.04$) than after gonadectomy (78.5; range 11.5–126.3; Fig. 1). Receiver operating characteristic analysis revealed a cut-off value of 6.5 for the urinary FSH to creatinine ratio to verify the presence or absence of functional testicular tissue.

The median urinary FSH to creatinine ratio in male dogs before deslorelin treatment was 0.4 (range 0–2.3), which was higher ($P < 0.01$) than during deslorelin treatment (Fig. 1). Follicle-stimulating hormone was not detectable in any of the urine samples from the male dogs during deslorelin treatment.

There was no correlation between the plasma and urinary FSH concentrations in bitches or in male dogs before gonadectomy or deslorelin treatment. However, in bitches there was a positive correlation between plasma and urinary FSH concentrations after gonadectomy ($P < 0.01$; correlation coefficient 0.70). In male dogs, there was no correlation between plasma and urinary FSH concentrations after gonadectomy or during deslorelin treatment.

4. Discussion

This is the first report of the validation of a method for measuring the concentration of FSH in canine urine. The accuracy of the validated assay is very high, even at dilutions as great as 1:128. The method provides an easy, noninvasive measurement of integrated FSH release in the dog.

In bitches, the difference in plasma FSH concentration before and after gonadectomy was highly significant, without overlapping of the ranges. Moreover, the difference in urinary FSH to creatinine ratios before and after gonadectomy was also highly significant and without overlapping of the ranges. Receiver operating characteristic analysis revealed a cut-off value of 2.9 with 100% specificity and 100% sensitivity, implying that a urinary FSH to creatinine ratio below 2.9 indicates the presence of functional ovarian tissue. Plasma FSH concentration can vary over time because of the pulsatile secretion of this hormone: during the luteal phase and anestrus, there are one or two LH and FSH pulses every 6 hours [4]. The urinary FSH to creatinine ratio is less affected by the pulsatile pattern of FSH secretion and is therefore more stable over time, which makes the results more reliable.

Little is known about longitudinal variation in plasma FSH concentration in bitches after gonadectomy, but Reichler et al.[11] (2004) found a decrease in plasma concentrations of LH and FSH, with a nadir at 10 weeks after ovariectomy. After this decrease, the plasma concentrations of the gonadotropins increased again and became stable at around 29 weeks after gonadectomy. Data on urinary FSH concentration during this period are lacking but given the slow decline (over weeks) and then a rise in plasma FSH during this period, it can be expected that changes will also be observed in urinary FSH levels. In the present study, plasma and urine samples were collected about 5 months after gonadectomy. It is possible that in some bitches, plasma FSH had not yet reached a stable high-basal level because of the lack of negative feedback of gonadal steroids after ovariectomy. In spite of this, the urinary FSH to creatinine ratio was much higher after ovariectomy than before, with a high sensitivity and specificity which emphasizes the robustness of this test. Our results suggest that measuring the urinary FSH to creatinine ratio is a reliable alternative to measuring plasma FSH concentration to verify the presence of functional ovarian tissue in bitches. Also, it is preferable to the use of plasma measurements because it involves a single sample, collecting urine is easier and is noninvasive.

Ascertaining the presence of remnant ovarian tissue (ROT) is more complicated because the HPG axis is often altered in bitches with ROT [9]. More research is needed to determine whether measurement of the urinary FSH to creatinine ratio can be helpful in detecting remnant ovarian tissue.

The basal plasma FSH concentration in the intact male dogs used in this study was lower than that in anestrous bitches. Also in other studies, basal plasma FSH concentration was found to be lower in male dogs than in bitches, although the difference was not significant [13,22]. This might be due to the relatively low number of male dogs used in these studies ($n = 5$ and $n = 14$) or to the high variability of plasma FSH concentrations [13,22], but the true cause remains unknown. Basal plasma estradiol concentrations in male dogs were previously reported to be higher than in anestrous bitches [13]. This may explain the lower plasma FSH concentration found in these male dogs because estradiol exerts a negative feedback on the hypothalamus and results in a decrease in kisspeptin mRNA. Hence, there is a decrease in GnRH release and consequently also in plasma LH and FSH concentrations [2,3].

As in the bitches, in male dogs, plasma FSH concentrations and urinary FSH to creatinine ratios were higher after gonadectomy than before gonadectomy, with no overlapping of ranges. This was also reported in another study concerning basal plasma FSH concentrations before and after gonadectomy [13]. Despite the lack of correlation between plasma and urinary FSH concentrations, the urinary FSH to creatinine ratio was much lower before gonadectomy than after and the cut-off value was 6.5 with 100% specificity and 100% sensitivity. The urinary FSH to creatinine ratio is therefore a reliable alternative to measurement of the plasma FSH and/or testosterone concentration for detection of functional testicular tissue.

In male dogs treated with the slow-release GnRH analog deslorelin, there is large individual variability in both the onset and the duration of action [17]. In some cases, it may be desirable to verify the level of suppression of the HPG axis, as for example, to determine whether a male dog is still infertile. The basal plasma FSH concentration was usually significantly lower during deslorelin treatment than before treatment, but the ranges overlapped. Urinary FSH was undetectable in all 13 dogs during deslorelin treatment, at 117 to 174 days after deslorelin administration. However, three of the dogs also had no detectable urinary FSH before treatment. Hence, neither the basal

plasma FSH concentration nor the urinary FSH to creatinine ratio can be used to assess the level of suppression of the HPG axis in male dogs treated with deslorelin.

The results of the pilot study concerning the storage conditions indicate that after its collection, the urine sample can be safely stored at −80 °C. Other studies have also found a decline in gonadotropin immunoreactivity in human urine stored at −20 °C but not in that stored at 4 °C or −80 °C [23,24]. In contrast, creatinine has been shown to remain stable in urine under different storage conditions, including at −20 °C, for at least 90 days [25–27], and in one study, urine creatinine was stable for more than 10 years [25].

In conclusion, the plasma FSH concentration and the urinary FSH to creatinine ratio were much higher in gonadectomized male and female dogs than in intact dogs, without overlapping of ranges. Both can be used reliably to verify the presence of functional gonadal tissue in dogs. However, collection of urine is easier, noninvasive, a single sample is enough and may thus be considered preferable. The plasma FSH concentration and the urinary FSH to creatinine ratio cannot be used for assessment of the level of HPG axis suppression in male dogs treated with deslorelin.

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