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Immunohistochemical localization of androgen and oestrogen receptors in canine hair follicles

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Abstract Skin biopsies from seven different body sites were obtained from 21 dogs of different breeds (short, normal, long hair), which were presented for euthanasia. Commercially available polyclonal antibodies were used for the immunohistochemical detection of androgen and oestrogen receptors. Both receptors showed a similar distribution in canine skin, with specific intranuclear staining. In the epidermis, the percentage of androgen receptor (AR)-positive cells, but not that of oestrogen receptor (ER)-positive cells, was significantly higher in samples from the thorax and the flank. In the dermal papilla, the percentage of ER-positive (but not AR-positive) cells was significantly lower in biopsies from the flank. No significant difference was found for both receptors between the locations in the outer root sheath, among the three different hair types, between sex and between intact and castrated dogs.

Keywords: androgen, canine, dermal papilla, dog, epidermis, hair follicles, immunohistochemistry, oestrogen, receptor, steroid.

INTRODUCTION

Canine endocrine alopecia is not fully understood. In addition to the quite well-defined conditions of alopecia occurring with hypothyroidism or with an oestrogen-producing Sertoli-cell tumour in male dogs, in which abnormal plasma concentrations of hormones can be measured, there are many clinical presentations of canine alopecia, the pathophysiology of which remains rather obscure. Attempts have been made to find abnormal concentrations of steroids or growth hormone in the plasma of affected dogs either through direct measurement or through stimulation tests.[1–3] Although reliable results have yet to be obtained,[4] steroid hormones in particular are still thought to play a major role in influencing canine hair growth. One of the typical signs of the so-called 'sexual-steroid associated dermatopathy' is hair loss at predilection areas of the flank or the perineum, whereas hair growth in other areas of the body, such as the head and limbs, is not affected. Neither the cause for this termination of hair growth, nor the reason for the hair loss occurring only at predilection areas is known. Peripheral conversion of steroid hormones within the cells of the skin and the hair follicles (as it is known in human androgenetic alopecia[5]) may reduce the effectiveness of measuring blood hormone concentrations. Variations in hormone receptor expression at different body sites,[6] is another hypothesis for the pathophysiology of hair loss restricted to predilection areas.

The objectives of this study were to demonstrate the specific localization of oestrogen receptors (ER) and androgen receptors (AR) in canine skin and hair follicles by immunohistochemistry and to compare the percentage of receptor-expressing cells in the interfollicular epidermis, the outer root sheath and the dermal papilla of the hair follicles at different body sites.

METHODS

Tissue samples

Skin samples were obtained from 21 dogs which were presented for euthanasia to local veterinarians. The age of the animals ranged from 8 months to 18 years (median 11 years). According to the hair type,[7] they were allotted to three groups: group 1, normal coat (nine German Shepherd Dogs); group 2, short coat (two Rottweiler, one Magyar-Viszla, one Podenco, one Weimaraner, one Wire-Haired Dachshund); group 3, long coat: (two Yorkshire Terriers, one Lhaso-Apso, one Irish Setter, one Kerry-Blue Terrier, one Collie-mix).

Skin biopsies (2×0.5 cm) were taken within 30 min of euthanasia from seven defined body sites (head, lateral side of the thorax, abdomen, flank, back, perineum, shank = craniolateral side of the tibia), fixed in

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4% buffered formaldehyde for 24 h and embedded in paraffin wax. From these tissue blocks 4-μm sections were cut. Control sections were stained with haematoxylin and eosin in order to detect any histopathological abnormalities. Only healthy skin was included in this study.

Immunohistochemical detection of androgen (AR) and oestrogen receptor (ER)

Sections for immunohistochemical analyses were placed on a poly L-lysine drop on APES-coated slides and air-dried for 90 min at 65 °C. Endogenous peroxidase activity was blocked with methanol/H_2O_2 and antigen retrieval was achieved using microwave heating in citrate buffer (pH 6.0) for 4×5 min (AR) or 2×5 min (ER). After cooling for 20 min at room temperature, sections were rinsed with phosphate-buffered saline (PBS, pH 7.4) and incubated with 1.5% goat serum (Vector Laboratories, Burlingame, CA, USA). Commercially available polyclonal antibodies were used for receptor detection as follows: AR, dilution 1:100 (ABR Affinity Bioreagents Inc., Golden, CO, USA) and ER, dilution 1:200 (Zymed Laboratories, San Francisco, CA, USA). Sections were incubated with the primary antibodies overnight at 4 °C, rinsed with PBS and incubated with Rabbit Dako EnVision system™ (Dako, Carpinteria, CA, USA). Antibody binding was detected with the chromogen DAB (Sigma-Aldrich, Wien, Austria) in Tris buffer (pH 7.4), rinsed with tap water and counterstained with Mayer's haematoxylin.

Tissue sections from human prostate were used as positive controls for the AR, and canine and bovine uterus were used for ER detection. In addition, bovine salivary gland tissue was used as combined positive and negative control for ER, as only epithelial cells of ducts were positive. Within the skin sections, sebaceous glands served as internal positive control for both antibodies.[8] As a negative control of the method, the first antibody was replaced by PBS. In addition, immunoprecipitation was performed by treating serial sections with normal rabbit IgG at the same protein concentration as the primary antibody followed by the EnVision kit™.

Evaluation

Positive and negative cell nuclei were counted using a ×25 objective, the microscope being combined with a computer-assisted image processing system (Metreo KAPPA ImageBase, Gleichen, Germany). Counting was performed in a double-blind study design. Three randomly selected areas were examined and the counts averaged. The percentage of positive nuclei was determined and used as dependent variable. Distributions did not differ notably from the normal distribution so that the General Linear Model (GLM) was used for statistical analysis (proc GLM; SAS Institute Inc., Release 8.01). To analyse the epidermis cells we used sex, breed and location (dog within sex and breed was included as a random factor) as independent variables. The independent variables for the analysis of the outer root sheath cells were sex, breed, location and the castration status (dog within sex, breed and castration status as a random factor). For the model analysing the dermal papilla cells the independent variables were sex, breed and location (dog within sex and breed as a random factor).

The Tukey–Kramer test was applied, in order to test which of the means would be significantly different between the body sites. The α level was set at 5%.

RESULTS

Both receptors showed a similar distribution in canine skin. Specific intranuclear staining could be detected in the keratinocytes of the interfollicular epidermis (Fig. 1a), in all layers of the sebaceous glands, in the cells of the arrector pili muscles, in endothelial cells of blood vessels, nerves and in some dermal fibroblasts.

Within the hair follicle, receptors were present in the outer root sheath (Fig. 1b), the hair matrix cells, the dermal papilla (Fig. 1c) and the connective tissue sheath. Slight to moderate cytoplasmatic staining was observed for both receptors in areas with high proliferative activity such as the hair matrix cells and the proximal parts of the outer root sheath of anagen hair follicles.

The percentage of positive stained cells was determined in the interfollicular epidermis, the transient part of the outer root sheath (the proximal part in telogen follicles, respectively) and in the dermal papilla.

No marked difference was found between the three groups of hair type, sex and intact or castrated dogs for both AR- and ER-positive cells.

Epidermis

Statistical analysis of AR in the epidermis revealed a significant difference ($P = 0.001$) between different body sites (Fig. 2), whereas there was no significant difference in the percentage of ER-positive nuclei. A significant difference was detectable in the following body sites (Tukey–Kramer test): head/thorax ($P = 0.0016$), thorax/back ($P = 0.027$), thorax/perineum ($P = 0.022$) (Table 1).

To evaluate receptor-positive cells in the outer root sheath and the dermal papilla we decided to restrict the number of locations to the four most relevant ones

Table 1. Mean and 95% confidence interval (CI) of the percentage (%) of androgen receptor-positive cells in the epidermis of biopsies from seven different skin locations

Location	LS-Mean	95% CI	
head	61.6	59.8	63.4
thorax	66.5	64.8	68.1
abdomen	62.9	61.0	64.7
flank	65.1	63.1	67.1
back	62.5	60.6	64.4
perineum	62.4	60.6	64.3
shank	63.0	61.2	64.7

LS-Mean, least square mean.

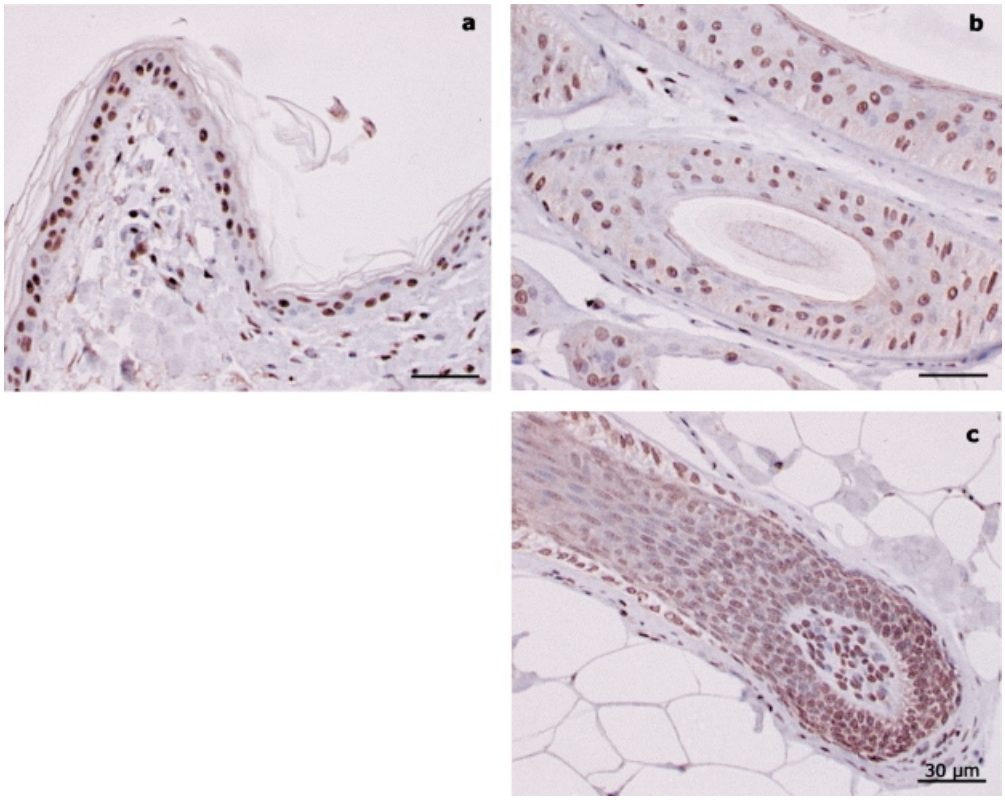

Figure 1. Immunohistochemical detection of oestrogen receptor (ER) in canine skin. Note positive and negative cell nuclei in (a) interfollicular epidermis (b) outer root sheath and (c) dermal papilla.

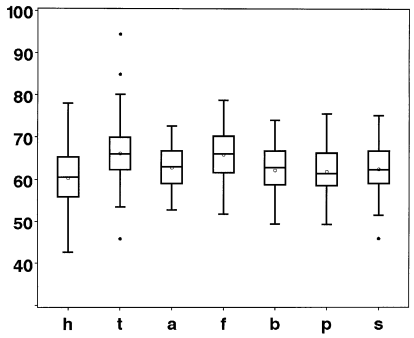

Figure 2. Boxplot of the percentage (%) of androgen receptor (AR)-positive cells in the epidermis of biopsies from seven different skin body sites: h, head; t, thorax; a, abdomen; f, flank; b, back; p, perineum; s, shank (craniolateral tibia).

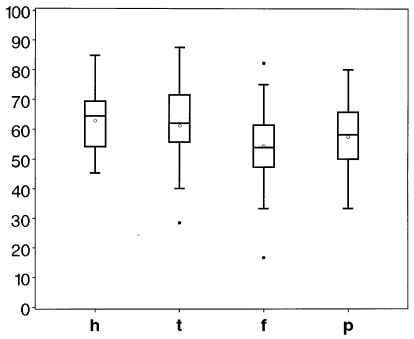

Figure 3. Boxplot of the percentage (%) of oestrogen receptor (ER)-positive cells in the dermal papilla of biopsies from four different skin body sites: h, head; t, thorax; f, flank; p, perineum.

(head, thorax, flank, perineum), which were used in another investigation (Bratka-Robia *et al.* in preparation), keeping whole hair follicles in culture for 7 days.

Outer root sheath

Statistical analysis showed that there was no significant difference in the percentage of receptor-positive cells among the locations, sex, breed and castration status and there was no significant interaction of sex × castration status (ER: $n = 185$; AR: $n = 182$).

Dermal papilla

Statistical analysis showed that there was no significant difference in the percentage of AR-positive cells ($n = 133$), whereas that of ER-positive cells ($n = 149$) was significantly ($P = 0.01$) different in the tested locations (Tukey–Kramer test) (Fig. 3).

The confidence interval of head and flank did not overlap, which means that head and flank differed significantly ($P = 0.007$) (Table 2).

Table 2. Mean and 95% confidence interval (CI) of the percentage (%) of oestrogen receptor-positive cells in the dermal papilla of biopsies from four different skin locations

Location	LS-Mean	95% CI	
head	63.8	60.2	67.3
thorax	61.6	57.5	65.9
flank	54.2	49.5	58.9
perineum	59.2	55.1	63.4

LS-Mean, least square mean.

A graphical display showed a slight difference of AR-positive cells between castrated and noncastrated dogs. Therefore, an additional model ($n = 133$) was calculated [independent variables were sex, location, castration status and the interaction sex × castration status; dog (within sex and castration status) was included as a random factor] but did not show a significant difference or a significant interaction.

DISCUSSION

The results of this study indicate that canine skin and hair follicles are target organs of sexual steroids. The presence and wide distribution of ER and AR in the interfollicular epidermis, outer root sheath and dermal papilla cells in canine skin is in contrast to immunohistochemical reports by other authors. This is the first report of quantitative results of the immunohistochemistry of the steroid receptors. Interestingly, there was no relevant difference in the percentage of receptor-positive cells between the predilection areas of alopecia and the other body sites. Only in the epidermis, was the percentage of AR-positive (but not ER-positive) cells significantly higher in samples from the thorax and the flank. In the dermal papilla the percentage of ER-positive (but not AR-positive) cells was significantly lower in biopsies from the flank.

Oh and Smart[9] detected ER in mouse skin almost exclusively in the dermal papilla, just as Mecklenburg[10] found in canine skin. Wallace and Smoller,[8] studying human skin, reported positive ER immunoreactivity in eccrine duct and sebaceous gland cells, as well as in some dermal papilla cells, but not in keratinocytes.

Only weak AR immunoreactivity could be detected in the interfollicular epidermis and in few keratinocytes of the outer root sheath, whereas cells of the dermal papilla showed distinct immunoreactivity throughout all phases of the hair cycle.[10] However, Diani and Mills[11] reported a similar distribution of AR immunoreactivity in the skin and hair follicles of the stumptailed macaque as we found in dogs.

The difference in the receptor distribution between our study and other reports, especially that of Mecklenburg,[10] may originate, on the one hand, in different fixation and antigen retrieval methods,[12] and, on the other hand, in the choice of the primary antibody. Although monoclonal antibodies were used by other authors,[10,11,13] we chose polyclonal antibodies in order to be able to cover the whole spectrum of the two receptor families with their isoforms (A and B). A difference in the expression of ER and AR subtypes in various compartments of the skin and the hair follicles is feasible.[14] Investigations of the distribution of steroid receptor subtypes in the canine skin have not been performed previously, but will be unavoidable in further research in the field of cutaneous manifestations of endocrine diseases.

With our control samples, we confirmed that the antibodies used for detection of ER and AR, which were raised against human antigens, cross-react sufficiently with canine and bovine antigens in a specific manner. However, immunohistochemical methods are only able to measure the presence of an antigenic epitope rather than the functional ability to bind the specific hormone.

Concerning the ER, the wide distribution in canine skin and hair follicles becomes reasonable, when the histopathological changes in sex hormone-associated alopecia are taken into consideration. Scott *et al.*[7] described in such cases orthokeratotic hyperkeratosis, epidermal atrophy, follicular atrophy, epidermal melanosis, follicular keratosis, dilatation, atrophy and telogenization of hair follicles, as well as sebaceous gland atrophy.

If the dermal papilla was the main pacemaker of the hair growth cycle, as proposed by Randall,[15] the growth cycle arrest of the hair follicle in the telogen phase can be explained. The atrophy of the follicle could be due to the lack of growth factors secreted by the dermal papilla cells. However, sebaceous gland atrophy and epidermal atrophy can hardly be explained as sole effects of alterations in dermal papilla signalling.

Androgen receptor

Androgens are the most investigated effector hormones of hair growth in the human, as they seem to play a major role in the development of androgenetic alopecia. AR were detected in dermal papilla cells and the connective tissue sheath of the human hair follicle, as well as in the interfollicular epidermis and the sebaceous glands. There were no AR in the epidermal cells of the hair follicle.[13] Sawaya and Price[16] found more intensive staining of the AR in the outer root sheath of human anagen hair follicles than in dermal papilla cells.

The role of androgens in the regulation of canine hair growth is not known. A testosterone-responsive alopecia was described by Scott *et al.*[7] but its pathomechanism is not clear. An indication of an effect of androgens is derived from the clinical observation of a change in the hair coat after castration of male dogs or caused by an inherited hypogonadism. Mecklenburg[10] found immunoreactivity of the AR in the dermal papilla cells throughout all phases of the hair cycle and strongest activity in the anagen phase. Weak immunoreactivity was observed in the keratinocytes of the interfollicular epidermis. The keratinocytes of the outer root sheath were AR-negative. In contrast to this, our results showed stronger immunoreactivity of the AR in the keratinocytes of the interfollicular epidermis and the outer root sheath.

Oestrogen receptor

The role of oestrogens in the regulation of canine hair growth is still speculative and based on clinical observations.[7] There is only one study, by Eigenmann *et al.*,[6] which relates the high concentration of ER in the affected skin region to the development of alopecia. Only one of six ovariectomized bitches, which received an oestradiol-17β implant reacted with bilateral symmetric alopecia

in the flank within 2 weeks. The concentration of ER in the periphery of the affected area was six times that in the centre or in control areas of the same or other bitches. However, 6 weeks after withdrawal of the implant, normal hair growth was resumed. The concentration of oestradiol-17β in blood was within the normal range of 104 ± 15 pg mL^{-1} in all six animals. The authors concluded that the high concentration of ER rather than a systemic hyperoestrogenism was causing the alopecia.

Mecklenburg[10] investigated the distribution of ER in skin biopsies of three body sites (shoulder, flank, caudal back) of eight healthy Beagles. He found ER-positive cells in the dermal papilla, especially in the catagen and telogen phase of the hair cycle. Our results show a wider distribution of the ER: interfollicular epidermis, dermal papilla, outer root sheath, matrix and arrector pili muscle cells. The reason for this discrepancy may be that Mecklenburg used a monoclonal antibody, whereas we used a polyclonal one. The advantage of a polyclonal antibody for such basic investigations is that a broader spectrum of epitopes on the receptor molecules can be recognized. It may also be that different isoforms of the ER (α or β) were present in different cells of the hair follicle. This may also be the case in the outer root sheath, where Mecklenburg[10] found only few ER-positive cells.

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REFERENCES

1. Rhodes, K.H. Cutaneous manifestations of canine and feline endocrinopathies. *Problems in Veterinary Medicine* 1990; **2**: 617–26.
2. Rosychuk, R.A.W. Cutaneous manifestations of endocrine disease in dogs. *Compendium on Continuing Education for the Practising Veterinarian* 1998; **20**: 287–302.
3. Schmeitzel, L.P., Lothrop, C.D. Jr Hormonal abnormalities in Pomeranians with normal coat and in Pomeranians with growth hormone-responsive dermatosis. *Journal of the American Veterinary Medical Association* 1990; **197**: 1333–41.
4. Bond, R. Hair follicles drive me crazy! *Veterinary Dermatology* 1999; **10**: 81–2.
5. Eicheler, W., Happle, R., Hoffmann, R. 5 Alpha-reductase activity in the human hair follicle concentrates in the dermal papilla. *Archives of Dermatological Research* 1998; **290**: 126–32.
6. Eigenmann, J.E., Poortman, J., Koeman, J.P. Estrogen induced flank alopecia in the female dog: evidence for local rather than systemic hyperestrogenism. *Journal of the American Animal Hospital Association* 1984; **20**: 621–4.
7. Scott, D.W., Miller, W.H. Jr, Griffin, C.E. Sex hormone dermatoses. In: *Small Animal Dermatology*, 6th edn. Philadelphia: W.B. Saunders, 2001: 827–45.
8. Wallace, M.L., Smoller, B.R. Estrogen and progesterone receptors in androgenic alopecia versus alopecia areata. *American Journal of Dermatopathology* 1998; **20**: 160–3.
9. Oh, H.S., Smart, R.C. An estrogen receptor pathway regulates the telogen-anagen hair follicle transition and influences epidermal cell proliferation. *Proceedings of the National Academy of Sciences of the United States of America* 1996; **93**: 12525–30.
10. Mecklenburg, L. *Untersuchungen zur Bedeutung potentieller Haarzyklusmodulatoren in der Haut des Hundes unter besonderer Berücksichtigung endokriner Dermatopathien.* Dissertation, vet. med., Tierärztliche Hochschule Hannover, Germany, 1998.
11. Diani, A.R., Mills, C.J. Immunocytochemical localization of androgen receptors in the scalp of the stumptail macaque monkey, a model of androgenetic alopecia. *Journal of Investigative Dermatology* 1994; **102**: 511–14.
12. Shi, R.S., Key, M.E., Karla, K.L. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *Journal of Histochemistry and Cytochemistry* 1991; **39**: 741–8.
13. Choudhry, R., Hodgins, M.B., Van der Kwast, T.H. *et al.* Localisation of androgen receptors in human skin by immunohistochemistry: implications for the hormonal regulation of hair growth, sebaceous glands and sweat glands. *Journal of Endocrinology* 1992; **133**: 467–75.
14. Paech, K., Webb, P., Kuiper, G.G. *et al.* Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* 1997; **277**: 1508–10.
15. Randall, V.A. The use of dermal papilla cells in studies of normal and abnormal hair follicle biology. *Dermatologic Clinics* 1996; **14**: 585–94.
16. Sawaya, M.E., Price, V.H. Different levels of 5 alpha-reductase type I and II, aromatase, and androgen receptor in hair follicles of women and men with androgenetic alopecia. *Journal of Investigative Dermatology* 1997; **109**: 296–300.

Résumé Des biopsies cutanées ont été obtenues sur 7 sites différents, à partir de 21 chiens de races variées (poil court, poil normal, poil long), présentés pour euthanasie. Des anticorps polyclonaux, disponibles commercialement, ont été utilisés pour détecter par immunohistochimie les récepteurs aux androgènes et aux oestrogènes. Les deux types de récepteurs étaient distribués de façon similaire dans la peau des chiens, avec un marquage intranucléaire spécifique. Dans l'épiderme, le pourcentage de cellules positives pour le récepteur aux androgènes (AR), était statistiquement plus élevé que pour les prélèvements réalisés sur le thorax et sur le flanc .Ceci n'a pas été retrouvé pour les cellules positives au récepteur aux oestrogènes (ER). Dans la papille dermique, le pourcentage de cellules ER-positives (mais pas AR-positives) était significativement plus faible dans les biopsies du flanc. Aucune différence significative n'a été retrouvée pour les deux types de récepteurs au niveau de la gaine

folliculaire externe, ni en fonction des trois types différents de pelage, du sexe ou entre chiens castrés ou non castrés.

Resumen Se tomaron biopsias cutáneas de siete áreas corporales distintas de 21 perros de diferentes razas (pelo corto, normal, y largo), que habían sido presentados para eutanasia. Se utilizaron anticuerpos policlonales para la detección inmunohistoquímica de receptores de andrógenos y estrógenos. Ambos receptores mostraron una distribución similar en la piel canina con tinción intranuclear específica. En la epidermis, el porcentaje de células positivas a receptor de andrógenos (AR), pero no el de las células positivas a receptores de estrógenos (ER), fue significativamente superior en las muestras de área torácica y del flanco. En la papila dérmica, el porcentaje de células positivas a ER (pero no positivas a AR) fue significativamente inferior en biopsias del flanco. No se encontraron diferencias significativas para ambos receptores entre las localizaciones en la baina externa de la raíz, entre los tres diferentes tipos de pelo, entre sexos y entre perros castrados y enteros.

Zusammenfassung Hautbiopsien von sieben verschiedenen Körperstellen wurden von 21 Hunden verschiedener Rasse (kurz-, normal- und langhaarig) genommen, die zur Euthanasie vorgestellt wurden. Kommerziell verfügbare polyklonale Antikörper wurden zur immunhistochemischen Identifizierung von Androgen- und Östrogenrezeptoren verwendet. Beide Rezeptoren zeigten eine ähnliche Verteilung in der Hundehaut mit spezifischer intranukleärer Anfärbung. In der Epidermis war der Prozentsatz der Androgen-Rezeptor (AR)-positiven Zellen aber nicht der von Östrogen-Rezeptor (ÖR)-positiven Zellen, bei Proben von Thorax und Flanke signifikant höher. In den Hautpapillen war der Prozentsatz der ÖR-positiven (aber nicht AR-positiven) Zellen in den von der Flanke entnommenen Proben signifikant niedriger. Zwischen den Rezeptoren der äusseren Haarscheide an den unterschiedlichen Köprerstellen, den unterschiedlichen Haartypen, dem Geschlecht und intakten und kastrierten Hunden bestand kein Unterschied.