New Insights in the Functional Zonation of the Canine Adrenal Cortex

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Background: Current understanding of adrenal steroidogenesis is that the production of aldosterone or cortisol depends on the expression of aldosterone synthase (CYP11B2) and 11β-hydroxylase cytochrome P450 (CYP11B1), respectively. However, this has never been studied in dogs, and in some species, a single CYP11B catalyzes both cortisol and aldosterone formation. Analysis of the canine genome provides data of a single *CYP11B* gene which is called *CYP11B2*, and a large sequence gap exists near the so-called *CYP11B2* gene.

Objectives: To investigate the zonal expression of steroidogenic enzymes in the canine adrenal cortex and to determine whether dogs have 1 or multiple *CYP11B* genes.

Animals: Normal adrenal glands from 10 healthy dogs.

Methods: Zona fasciculata (zF) and zona glomerulosa (zG) tissue was isolated by laser microdissection. The mRNA expression of steroidogenic enzymes and their major regulators was studied with RT-qPCR. Southern blot was performed to determine whether the sequence gap contains a *CYP11B* gene copy. Immunohistochemistry (IHC) was performed for 17α-hydroxylase/17,20-lyase (CYP17).

Results: Equal expression ($P = .62$) of the so-called *CYP11B2* gene was found in the zG and zF. Southern blot revealed a single gene. *CYP17* expression ($P = .05$) was significantly higher in the zF compared with the zG, which was confirmed with IHC.

Conclusions and Clinical Importance: We conclude that there is only 1 *CYP11B* gene in canine adrenals. The zone-specific production of aldosterone and cortisol is probably due to zone-specific CYP17 expression, which makes it an attractive target for selective inhibition of cortisol synthesis without affecting mineralocorticoid production in the zG.

Key words: CYP11B1; CYP11B2; CYP17; Steroidogenesis.

In the canine adrenal cortex, 3 functional and morphological layers can be distinguished: the outermost zona glomerulosa (zG), the middle zona fasciculata (zF), and the inner zona reticularis (zR).[1,2] The zG is responsible for the production of the mineralocorticoid aldosterone, which is regulated by the plasma potassium, angiotensin II, and ACTH concentration. The zF and zR function together as a unit and are the main source of body's glucocorticoid secretion, which is strictly regulated by the hypothalamic-pituitary-adrenocortical axis. Additionally, the zR may also produce androgens such as dehydroepiandrosterone and androstenedione. Using cholesterol as a uniform precursor molecule, zone-dependent enzyme expression is essential for distinct steroid production.[1–4]

Abbreviations:

3βHSD	3β-hydroxysteroid dehydrogenase
AGTR2	angiotensin II receptor 2
BAC	bacterial artificial chromosome
BSA	bovine serum albumin
CT	cycle threshold
CYP11A1	cytochrome P450 side chain cleavage
CYP11B1	11β-hydroxylase cytochrome P450
CYP11B2	aldosterone synthase
CYP17	17α-hydroxylase/17,20-lyase
CYP21	21-hydroxylase
DAB2	disabled-2
ECL	enhanced chemiluminescence
MC2R	ACTH receptor
NCBI	National Center for Biotechnology Information
PR	progesterone receptor
RPS19	ribosomal protein S19
RPS5	ribosomal protein S5
SDHA	succinate dehydrogenase complex subunit A
SF-1	steroidogenic factor 1
SPIA	single primer isothermal amplification
SPRP	small proline rich protein
StAR	steroidogenic acute regulatory protein
TBST	tris buffered saline with tween
Wnt4	wingless-type MMTV integration site family, member 4
zF	zona fasciculata
zG	zona glomerulosa
zR	zona reticularis

In humans, rats, and mice, the last steps in aldosterone and cortisol/corticosterone synthesis are catalyzed, respectively, by 2 different but related enzymes: the zG-specific aldosterone synthase (CYP11B2) and zF (and zR)-specific 11β-hydroxylase cytochrome P450

(CYP11B1) (Fig 1).[5–7] In contrast, cows,[8] pigs,[9] sheep,[10] birds, and amphibians[11,12] have only CYP11B1 to catalyze both cortisol and aldosterone formation.

Because humans and dogs have the same adrenocortical end products, ie, aldosterone and cortisol, human and canine steroidogenesis was assumed to be identical. Therefore, in current veterinary literature, the published cascade of canine adrenocortical steroidogenesis is mainly a human variant.[1,13,14] However, according to the National Center for Biotechnology Information (NCBI) database, the canine genome does contain a *CYP11B2* (NC_006595.3) gene, but no *CYP11B1* can be found. Near this so-called *CYP11B2* gene on chromosome 13 lies a large sequence gap. The *CYP11B* gene duplicates in humans and mice lie very close to each other,[6] so this sequence gap could be the place for another canine *CYP11B* gene copy. The question is whether the dog has both *CYP11B1* and *CYP11B2*, or if the only canine *CYP11B* gene has erroneously been denominated *CYP11B2*.

In animals with both CYP11B1 and CYP11B2, zone-specific expression of these 2 enzymes can explain zone-dependent steroidogenesis. In animals with only 1 CYP11B, other enzymes must have a zone-dependent expression to maintain zone-specific steroidogenesis.[8,11,12,15] In humans (Fig 1), 17α-hydroxylase/17,20-lyase (CYP17) is known to play an important role in functional zonation: it is absent in the zG which facilitates aldosterone production, it is present in the zF in which it predominantly executes its 17-hydroxylase activity necessary for cortisol production, and it is also present in the zR in which it is a cofactor for adrenal androgen production through its 17,20-lyase reaction.[16] Adult rats, mice, rabbits, and hamsters do not express CYP17, and therefore, corticosterone, and not cortisol, is the end product in their glucocorticoid pathway.[3,17,18]

The aim of this study was to study the number of *CYP11B* genes in the canine genome and to determine which (other) enzymes are zone specific in the canine adrenal cortex. We, therefore, studied (1) gene expression in isolated zF and zG tissue to determine the relative mRNA expression of steroidogenic enzymes and their major regulators: the adrenocorticotropic hormone receptor (*MC2R*) and steroidogenic factor 1 (*SF-1*), and (2) the large sequence gap near the so-called *CYP11B2* gene on chromosome 13 to determine whether dogs have 1 or multiple *CYP11B* genes.

Materials and Methods

Animals and Tissues

For this study, normal adrenal glands were used, obtained from 10 healthy dogs, of which 5 dogs were used for immunohistochemistry (IHC) and the other 5 dogs for microdissection. The dogs were euthanized for reasons unrelated to this study, approved by the Ethical Committee of Utrecht University. All dogs were of mixed breed from 2 to 6 years of age, all dogs were intact, 6 dogs were female, and 4 dogs were male.

For IHC, adrenal glands from 5 dogs were fixed in 4% buffered formalin. After 24–48 hours of fixation, the tissues were embedded in paraffin and cut into 5-μm sections, after which they were mounted on SuperFrost Plus microscope slides.[a] For microdissection, fresh tissue from adrenal glands from 5 dogs was snap frozen in liquid nitrogen within 10 minutes after resection and stored at −70°C until further use.

Microdissection

Cryosections of 8 μm were made, and 5–8 sections were mounted on an MMI MembraneSlide. The slides were directly processed or stored at −70°C, for a week at maximum. To ensure that enough RNA was left in the frozen sections, 1 section was directly put in RLT plus buffer (QIAGEN Venlo, The Netherlands) and stored in a −20°C freezer until RNA isolation. The frozen sections from the −70°C freezer were defrosted at room temperature, quickly fixed in 70% isopropyl alcohol and stained with 1 : 1 hematoxylin for correct orientation of the tissue. The

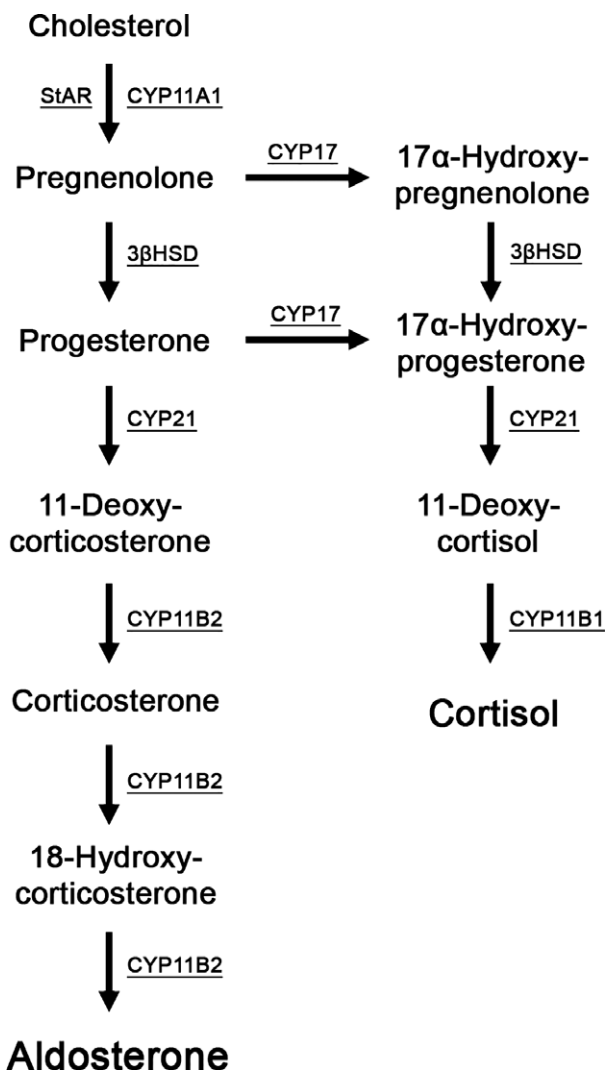**Fig 1.** Human adrenocortical steroidogenesis in the zona glomerulosa and zona fasciculata. StAR = steroidogenic acute regulatory protein, CYP11A1 = cytochrome P450 side chain cleavage, 3βHSD = 3β-hydroxysteroid dehydrogenase, CYP17 = 17α-hydroxylase/17,20-lyase, CYP21 = 21-hydroxylase, CYP11B1 = 11β-hydroxylase cytochrome P450, CYP11B2 = aldosterone synthase. Adapted from Payne and Hales[6] and Pihlajoki et al.[37]

sections were quickly washed with milliQ and dehydrated with 70% isopropyl alcohol, 100% isopropyl alcohol, and xylene. Areas of zG and zF were cut with Laser Microdissection (MMI CellCut) with a Nikon (Amsterdam, The Netherlands) Eclipse TE300 microscope, 10^6 μm^2 per zone. To ensure that exclusively the desired zone was cut, areas from the zG were cut from the center of the zG and areas from the zF were cut from the center of the upper half of the zF. The zones were collected in tubes with sticky caps (MMI), and 60 μL RLT plus buffer (QIAGEN) was added to the tissue after which it was stored at −20°C. RNA isolation was carried out with the QIAGEN RNeasy Micro Kit according to protocol. RNA quality was analyzed with the BioAnalyzer Pico Total RNA Kit. NuGEN Ovation PicoSL WTA System V2 was used to amplify RNA. Single Primer Isothermal Amplification (SPIA, NuGEN) cDNA was stored at −20°C. The SPIA cDNA concentration was measured with NanoDrop.

Primer Design

All primers for qPCR were based on mRNA sequences of our genes of interest that were obtained from the NCBI GenBank database. Primers (Table 1) were designed using Perl-primer (v1.1.14: Copyright © 2003-2006 Owen Marshall, http://perlprimer. sourceforge.net/) software according to the parameters in the Bio-Rad (Veenendaal, The Netherlands) iCycler manual, checked for secondary structure formation with mfold web server v3.1, and ordered from Eurogentec (Maastricht, The Netherlands). Optimization and confirmation of primer specificity of the PCR reaction were performed as described previously.[19]

RT-qPCR

To determine and compare the expression levels of zG markers angiotensin II receptor 2 (AGTR2), Disabled-2 (DAB2), Wingless-type MMTV integration site family, member 4 (Wnt4), and progesterone receptor (PR), of steroidogenic factors MC2R and SF-1, and of steroidogenic enzymes steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage (CYP11A1), 3β-hydroxysteroid dehydrogenase (3βHSD), CYP17, 21-hydroxylase (CYP21), and CYP11B2 between the zG and the zF, RT-qPCR analyses were performed on 0.25 μg/μL amplified SPIA cDNA. Ribosomal protein S5 (RPS5), ribosomal protein S19 (RPS19),

Table 1. Primer pairs.

Target gene		Sequence (5′ → 3′)	Ta (°C)	Accession number	Length of product (bp)
AGTR2	For	GGT GCT ATT ACG TCC CCG AG	56.8	XM_549206.3	205
	Rev	CTG ATG GTG GCA AGG GTG AT			
DAB2	For	TGC AAC CCT ACA GAC CAA CC	62.0	XM_536493.4	179
	Rev	GAC CAC TCA TCA TGG CTC CC			
Wnt4	For	CGA GGA GTG CCA GTA CCA GT	61.0	XM_005617834.1	124
	Rev	AGA GAT GGC GTA CAC GAA GG			
PR	For	CAA TGG AAG GGC AGC ATA AC	58.5	NM_001003074.1	103
	Rev	CAG CAC TTT CTA GGC GAC A			
MC2R	For	TCA TGT GGT TTT GCC GGA AGA GAT	58.5	XM_003638756.1	138
	Rev	AAT GGC CAG GCT GCA AAT GAA A			
SF-1	For	AGG GCT GCA AGG GGT TTT TCA A	59.0	XM_846937.2	143
	Rev	CAT CCC CAC TGT CAG GCA CTT CT			
StAR	For	CTC TGC TTG GTT CTC GG	62.5	NM_001097542.1	125
	Rev	CCT TCT TCC AGC CTT CC			
CYP11A1	For	CAC CGC CTC CTT AAA AAG TAA CAA G	63.3	XM_535539.3	129
	Rev	GCT GCG TGC CAT CTC GTA G			
3βHSD	For	CAG GAG GGT TTC TGG GTC AG	56.5	NM_001010954	186
	Rev	AGG CTC TCT TCA GGC ACT GC			
CYP17	For	CCT GCG GCC CCT ATG CTC	60.0	XM_535000.3	134
	Rev	GGC CGG TAC CAC TCC TTC TCA			
CYP21	For	AGC CCG ACC TTC CCC TCC ACC TG	64.5	NM_001003335	152
	Rev	TCT GCC GGC GAA GTC CAC CCA TTT			
CYP11B2	For	GCC TAC CCC TTG TGG ATG AC	62.0	XM_00343180587.1	126
	Rev	CTC TGT GAC TGC TGT CTG GG			
RPS5	For	TCA CTG GTG AGA ACC CCC T	62.5	XM_533568	141
	Rev	CCT GAT TCA CAC GGC GTA G			
RPS19	For	CCT TCC TCA AAA AGT CTG GG	61.0	XM_005616513	95
	Rev	GTT CTC ATC GTA GGG AGC AAG			
SRPR	For	GCT TCA GGA TCT GGA CTG C	61.2	XM_546411	81
	Rev	GTT CCC TTG GTA GCA CTG G			
SDHA	For	GCC TTG GAT CTC TTG ATG GA	61.0	DQ402985	92
	Rev	TTC TTG GCT CTT ATG CGA TG			
Probe	For	GTC GCT ACA GGC CGT CCA GAA GT	63.7	XM_00343180587.1	307
CYP11B2	Rev	GCC CAG CCG CTC CCC GAA CAG			

Primer pairs for qPCR analysis of AGTR2, DAB2, Wnt4, PR, MC2R, SF-1, StAR, CYP11A1, 3βHSD, CYP17, CYP21, the so-called CYP11B2, and the reference genes RPS5, RPS19, SRPR, and SDHA, and a primer pair designed to create a probe for CYP11B1/2, positioned halfway into exon 3 through halfway into exon 4. All positions are based on canine mRNA sequences, as published in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/). For = forward primer; Rev = reverse primer; Ta = annealing temperature; bp = base pairs.

small proline-rich protein (*SPRP*), and succinate dehydrogenase complex subunit A (*SDHA*) were used as reference genes to correct for differences in cDNA concentration.[20–22] SYBRgreen supermix (Bio-Rad) was used for detection, and CFX Manager 3.0 (Bio-Rad) was used to analyze the data. GeNorm software (https://genorm.cmgg.be/) was used to analyze relative expression levels of the reference genes, which justified their use. The $2^{-\Delta\Delta Ct}$ method[23] was used to calculate the normalized relative expression of each target gene.

Southern Blot

The Bacterial Artificial Chromosome (BAC) clones used were CH2-265M08 (M08) and CH82-485G10 (G10), as ordered from the BACPAC Resources Center, Pieter de Jong's Laboratory, of CHORI-82. On the G10 BAC, the so-called *CYP11B2* is present. The M08 BAC contains both the so-called *CYP11B2* gene and the sequence gap. Both BACs were cultured on agar plates. Of the cultured bacteria, a colony pick was inoculated into LB-Miller medium with 1% chloramphenicol and kept at 37°C with agitation overnight. DNA was isolated with QIAGEN Maxi Kit, according to protocol. Both BACs were cut with restriction enzyme BamHI (Promega, Leiden, The Netherlands); the restriction site for BamHI is 5′ G↓GATCC3′ 3′ CCTAG↑G5′. Approximately 500 ng per BAC was used and electrophoresed on a 0.8% agarose TAE gel overnight. Southern blot was performed according to protocol of Davis et al.[24] A probe was made with the primers shown in Table 1, which were designed using PrimerSelect of DNASTAR Lasergene 12, and ordered from Eurogentec. SYBRgreen supermix (Bio-Rad) was used for amplification, and a temperature gradient was performed to determine the optimal annealing temperature. A C1000 Touch thermal cycler (BioRad) was used for all PCR reactions. The product was put on a 1% agarose TBE gel and checked for correct product size after electrophoresis, after which the DNA was isolated from the gel with QIAquick Gel Extraction Kit (QIAGEN) according to the protocol. The *CYP11B* probe was labeled with the Thermo Scientific Biotin DecaLabel DNA Labeling Kit according to the protocol. With the North2South Chemiluminescent Hybridization and Detection Kit, the southern blot membrane was hybridized overnight with the labeled probe of which attachment was subsequently detected with the same kit, according to the protocol.

Analysis Sequence Gap

In the canine genome as documented in the NCBI GenBank database, near to the so-called *CYP11B2* gene, a region of approximately 50,000 bp (Chr13: ±39,840,000–39,890,000) is presented as a sequence gap, which is present on BAC CH82-265M08 (M08). Of this M08 BAC, a draft sequence containing 36 unordered contigs, an unordered set of partly overlapping DNA segments, can be found under accession number AC183568.1. Using MegAlign from DNASTAR Lasergene 12, the contigs were aligned with the known M08 sequence, which left contigs corresponding to the sequence gap. Using the NCBI BLAST tool (blast.ncbi.nlm.nih.gov), these contigs were blasted against the canine so-called *CYP11B2* gene.

For analysis of the amino acid sequence of the known canine CYP11B enzyme, accession number XP_539192.3 was used.

Immunohistochemistry

The paraffin-embedded sections were rehydrated in a series of xylene and ethanol baths. Antigen retrieval was achieved by using a citrate buffer (0.01 M, pH 6.0, 0.05% tween) for CYP17 or Tris/EDTA (10 mM Tris base, 1 mM EDTA, 0.05% tween, pH 9.0) for Wnt4 at 98°C for 10 minutes. The slides were immersed in 3.5% (CYP17) or 0.35% (Wnt4) H_2O_2 in Tris buffered saline with 0.1% tween (TBST0.1%, 0.02 M, pH 7.6) for 30 minutes to block endogenous peroxidase; 5–10% normal goat serum (NGS) in 1% bovine serum albumin (BSA) was applied to the slides to block nonspecific binding sites. The slides were incubated with the anti-CYP17 antibody (courtesy of A.J. Conley, rabbit-anti-bovine, 1 : 7,500) or the anti-Wnt4 antibody (rabbit-anti-human, 1 : 6,000) and kept at 4°C overnight. The following day, secondary anti-rabbit antibody (Envision, K4003) was applied for 50–90 minutes. Bound antibody was visualized with the DAB substrate kit for peroxidase (DAKO, K3468, Heverlee, Belgium), after which the slides were counterstained with hematoxylin, dehydrated in a series of ethanol and xylene baths, and embedded with VectaMount Mounting Medium (H-5000).

Immunoprecipitation

For normal adrenal homogenate, protein was isolated from a normal canine adrenal gland using radioimmunoprecipitation (RIPA) buffer base. To isolate CYP17 protein out of normal adrenal homogenate, immunoprecipitation was performed with the anti-CYP17 antibody, with Dynabeads Protein A (Invitrogen, Life Science Technologies, Breda, The Netherlands). 250 μL of a normal adrenal gland homogenate (8.05 μg/μL) was incubated with 50 μL of 1 : 2,500 anti-CYP17 antibody; 20 mg of Protein A was dissolved in 200 μL phosphate-buffered saline and added to the homogenate/antibody solution. The solution was incubated for 30 minutes at room temperature and then centrifuged for 10 minutes at 1,000 rates per minute. The supernatant was removed and the sediment was washed with TBST0.1% and again centrifuged. Fifty microliters of phosphate/citrate pH 3 was added to the remaining sediment and the concoction was incubated for 15 minutes at room temperature, and then centrifuged for 5 minutes at 13,400 rate per minute.

Western Blot

To confirm specificity of the anti-CYP17 antibody, Western blot was performed using the immunoprecipitate from the previous paragraph (0.39 μg/μL), a normal adrenal homogenate (4 μg/μL), and a negative control containing only RIPA buffer base. All samples were diluted 1 : 1 with sample buffer with dithiothreitol and heated at 95°C for 2 minutes. A 10% acryl/bisacryl running gel was used; 20 μL of the diluted samples or 12 μL of the Precision plus Protein Standard (BioRad) was loaded onto the gel. After gel-electrophoreses, the gel was blotted onto a Hybond Enhanced Chemiluminescence (ECL) nitrocellulose membrane (Amersham, GE Healthcare, Diegem, Belgium). The membrane was blocked for 60 minutes in TBST0.1% with 4% ECL Blocking Agent, after which it was incubated overnight at 4°C in the anti-CYP17 antibody in a 1 : 2,500 concentration, diluted in TBST0.1% in 4% BSA. The next day, the membranes were incubated for 60 minutes with the secondary antibody (anti-rabbit, horseradish peroxidase conjugated, 1 : 20,000). TBST0.1% was used for all washing steps. An ECL advanced Western blotting detection kit (Amersham RPN2135, GE Healthcare) was used for protein visualization, and chemiluminescence was detected using a ChemiDoc XRS Chemi Luminescent Image Capture (BioRad).

Statistical Analyses

Statistical analyses were performed using SPSS22 (IBM, Armonk, NY). Due to the nonnormal distribution of the variables, mRNA expression levels between groups were compared with the nonparametric Mann-Whitney *U*-test. Relative

expression levels were compared between the zG and the zF, where the mean delta cycle threshold (CT) of all samples was used as reference point to calculate the $2^{-\Delta\Delta Ct}$.[23] A P value ≤.05 was considered significant.

Results

The relative mRNA expressions of *AGTR2* (P = .014), *DAB2* (P = .014), *Wnt4* (P = .05), and *PR* (P = .027) were significantly higher in the zG compared with the zF (Table 2), indicating successful separation of zG and zF tissue. Immunohistochemical staining confirmed the zG specificity of Wnt4 (Fig 2).

RT-qPCR analysis revealed equal expression of the so-called *CYP11B2* gene in both zG and zF (Table 2), which could suggest that there is only 1 *CYP11B* gene in the dog. To further prove this, we examined 2 BAC clones: 1 BAC containing the so-called *CYP11B2* gene, BAC G10; and 1 BAC containing the so-called *CYP11B2* gene plus the sequence gap, BAC M08. BamHI was used as restriction enzyme to cut the BACs. The predicted fragment size containing the so-called *CYP11B2* gene was 3,900 bp, based on the BAC's sequence information. Southern blot analysis of the BACs revealed a single restriction fragment which corresponded to the predicted fragment size that hybridized to the *CYP11B* probe in both BACs (Fig 3).

Two predicted protein isoforms can be found in the NCBI database corresponding to the canine so-called *CYP11B2* gene, but the names change to CYP11B1 on protein level. The amino acid sequences of these proteins do correspond to the so-called *CYP11B2* gene. Analysis of the amino acid sequences showed a proline at residue 112, a glycine at residue 288, and an alanine at residue 320.

Next, we analyzed the 36 unordered contigs from the BAC M08 to determine whether (parts of) an extra *CYP11B* gene was present in the sequence gap. Of the 36 unordered contigs, 14 contigs could be aligned along the sequenced parts of M08. This left the other contigs to belong to the sequence gap. When blasting the exons of the so-called *CYP11B2* gene against these gap contigs, exon 1 was found full length on contigs 11, 32, and 34 (97–99% similarity), approximately half of exon 2 (first 85 out of 156 bp) was found on the same contigs (96% similarity in all 3 contigs), a large part of exon 9 (1,262–1,264 out of 1,589 bp) was found on contigs 32 and 34 (92 and 91% similarity), and a small part of exon 9 (158 out of 1,589 bp) was found on contig 11 (86% similarity). On these 3 contigs, exon 2 was quickly followed by exon 9. Exons 3–8 were not found on any of the contigs belonging to the sequence gap.

The relative mRNA expressions of *StAR* (P = .014), *CYP11A1* (P = .014), and *CYP17* (P = .05) were significantly higher in the zF compared with the zG (Table 2). Immunohistochemical staining for CYP17 demonstrated a clear cytoplasmic granular pattern in the zF and the zR in all sections (Fig 4). In the capsule, the zG, and the medulla, the staining was absent. In both the normal adrenal homogenate and the immunoprecipitate, Western blotting for CYP17 revealed a band at approximately 50 kDa (Fig 5), corresponding to CYP17's expected weight.[25] The normal adrenal homogenate produced a more intense signal compared with the immunoprecipitate, which can be explained by the higher protein concentration in the normal adrenal homogenate. No band was visible in the negative control.

The relative mRNA expression of *3βHSD, CYP21, MC2R,* and *SF-1* did not differ significantly between the 2 zones (Table 2).

Discussion

This study is the first to demonstrate the zone-specific expression of steroidogenic enzymes in the canine adrenal cortex. Studying the expressions of *CYP11B1* and *CYP11B2* in the canine adrenal cortex was challenging,

Table 2. RT-qPCR results.

Gene	Mean FC zG	95% CI	Mean FC zF	95% CI	Ratio zG/zF	P
Zone-specific markers						
AGTR2	4.33	−0.07 to 8.72	0.29	−0.13 to 0.71	14.93	**.014**
DAB2	2.02	0.69–3.34	0.51	0.33–0.69	3.96	**.014**
Wnt4	7.11	0.09–14.12	0.73	−1.23 to 2.68	9.74	**.05**
PR	3.98	2.69–5.28	1.15	−1.41 to 3.71	3.46	**.027**
Steroidogenic factors						
MC2R	1.42	0.45–2.40	0.94	−0.36 to 2.25	1.51	.142
SF-1	1.23	0.47–1.99	0.98	0.24–1.72	1.26	.462
Steroidogenic enzymes						
StAR	0.77	0.36–1.18	1.61	1.05–2.16	0.48	**.014**
CYP11A1	0.49	0.13–0.85	2.85	2.31–3.38	0.17	**.014**
3βHSD	0.92	0.25–1.59	1.42	0.50–2.34	0.65	.221
CYP17	0.91	−0.85 to 2.67	4.26	0.14–8.38	0.21	**.05**
CYP21	1.23	0.54–1.91	1.08	−0.10 to 2.25	1.14	.624
CYP11B2	2.44	−0.37 to 5.25	1.72	0.13–3.31	1.42	.624

RT-qPCR results for *AGTR2, DAB2, Wnt4, PR, MC2R, SF-1, StAR, CYP11A1, 3βHSD, CYP17, CYP21,* and the so-called *CYP11B2*. FC = fold change, zG = zona glomerulosa, zF = zona fasciculata, CI = confidence interval. P values ≤.05 were considered significant and are emphasized in bold italic.
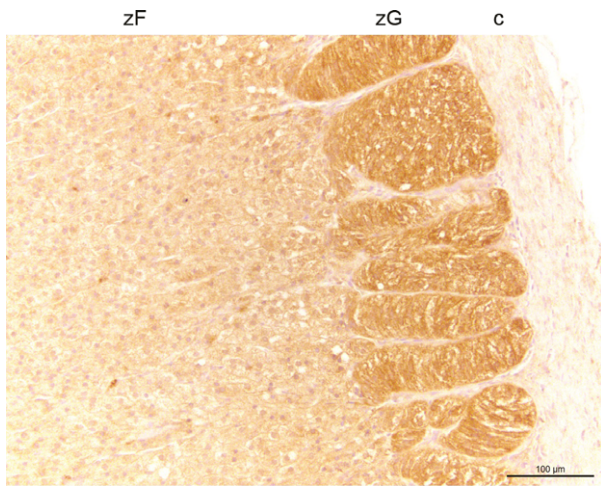
Fig 2. Immunohistochemical staining Wnt4. Representative example of immunohistochemical staining for Wnt4 in a normal adrenal gland. Staining is most intense in the zG in a cytoplasmic granular pattern. Staining is faintly visible in the zF and capsule. Bar represents 100 μm. c = capsule, zG = zona glomerulosa, zF = zona fasciculata.
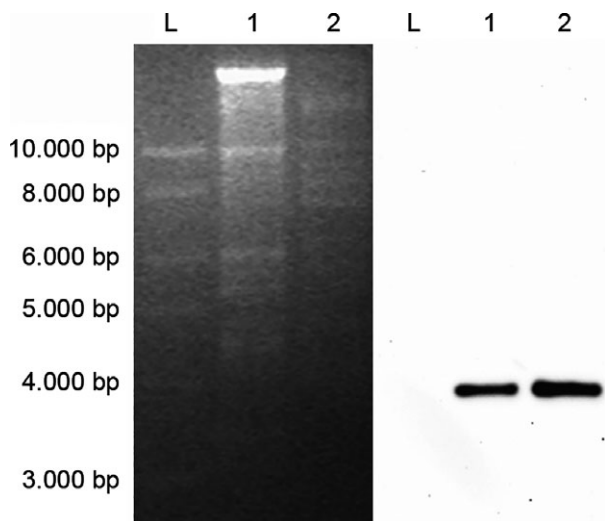

Fig 3. Southern blot. Left: EtBr staining of the gel. Right: After southern blot and hybridization. L: DNA ladder (Promega); lane 1: CH82-265M08 (M08) BAC; lane 2: CH82-485G10 (G10) BAC. Both BACs were cut with BamHI (Promega). Both BACs show only 1 signal at approximately 4,000 bp.

as the sequence of only 1 gene ("*CYP11B2*") is known. Our RT-qPCR results of the so-called *CYP11B2* showed equal expression in the zG and zF, suggesting that the canine adrenal cortex expresses only 1 *CYP11B* gene. The significantly higher expression of *AGTR2*,[26] *DAB2*,[27] *Wnt4*,[28] and *PR*[29,30] in the zG compared with the zF justified their use as canine zG markers and confirmed correct isolation of the respective zones. Equal *CYP11B2* distribution could thus not be attributed to incorrect zone isolation.

In the canine genome, near the known sequence of the so-called *CYP11B2* gene, a large sequence gap is present. Because the *CYP11B* gene duplicates in humans and mice lie very close to each other,[6] we questioned whether this sequence gap could be the place for another *CYP11B* gene copy. Southern blot results with a probe from exons 3 to 4 rejected this, as no complete *CYP11B* gene copy was present in the sequence gap on the M08 BAC. Hypothetically, an existent *CYP11B* gene copy could be mutated in a way that it still codes for a functional CYP11B enzyme, but it was not similar enough to the known *CYP11B* gene in our Southern blot probe to recognize it. Another option is that there is another *CYP11B* gene copy which is not located on the M08 BAC, but somewhere else in a nonsequenced part of the canine genome. However, this seems unlikely, since the gene duplicates in humans and mice are separated by no more than 40 kb and 8 kb, respectively.[6] A gene knock-out study (siRNA of the so-called *CYP11B2*) to analyze aldosterone and cortisol concentrations would likely produce a more definitive conclusion as to whether there is only 1 *CYP11B* gene in the dog.

Curnow et al.[31] state that the amino acid sequence of a CYP11B protein can predict whether the enzyme has an aldosterone synthase activity. They found that a CYP11B1 enzyme which has a serine to glycine substitution at residue 288 (Ser288Gly) and a Val320Ala is a fully functioning aldosterone synthase.[7,31] An Ile112Pro in human *CYP11B2* has been shown to give a 3-fold increase of 11β-hydroxylation activity and a 2-fold increase of 18-hydroxylation.[7] Analysis of the known canine *CYP11B* amino acid sequence showed the presence of both a glycine at residue 288 and an alanine at residue 320, thereby suggesting that the corresponding enzyme has aldosterone synthase activity. Furthermore, the presence of a proline at residue 112 in the known canine *CYP11B* suggests that this enzyme has a higher 11β-hydroxylase activity than the human CYP11B2, which is convenient for cortisol production. The combined findings imply that the known canine CYP11B concerns an enzyme which has aldosterone synthase activity but is also efficient in cortisol production.

Interestingly, our search for analogous exons of *CYP11B* in the sequence gap of the unordered contigs showed fragments of exons 1, 2, and 9, but not exons 3–8. The exon 2 fragment was closely or directly followed by the exon 9 fragment. Pseudogenes have been reported in, among others, guinea pigs,[32] rats,[33] and cows.[34] Rats have 4 *CYP11B* genes in total: *CYP11B1, CYP11B2, CYP11B3,* and *CYP11B4,* of which *CYP11B3* is very similar to *CYP11B1,* and *CYP11B4* is a pseudogene which misses exon 3 and part of exon 4. Cows have no less than 5 *CYP11B* genes,[34] of which 2 have similar enzymatic activities (both are called *CYP11B1*) and 3 are pseudogenes. The presence of exons 1, 2, and 9 in the sequence gap most likely indicates remnants of a canine *CYP11B* pseudogene.

Birds and amphibians possess only 1 *CYP11B* gene: *CYP11B1.*[11,12] This *CYP11B1* seems to be a common ancestral gene of which duplication and specialization is restricted to mammals and has occurred in at least
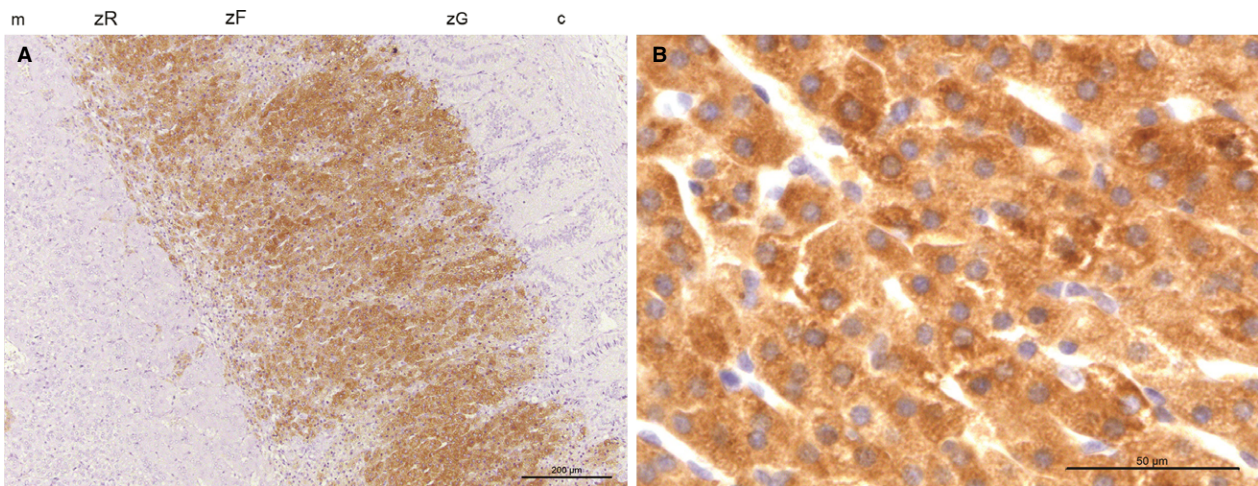
Fig 4. Immunohistochemical staining CYP17. Representative examples of immunohistochemical staining for CYP17 in normal adrenal glands. (**A**) CYP17 positive cells detected throughout the entire zF and zR. Staining is absent in the capsule, zG, and medulla. Bar represents 200 μm. (**B**) Magnification with a 40× objective of the zF, showing a cytoplasmic granular staining pattern. Bar represents 50 μm. c = capsule, zG = zona glomerulosa, zF = zona fasciculata, zR = zona reticularis, m = medulla.
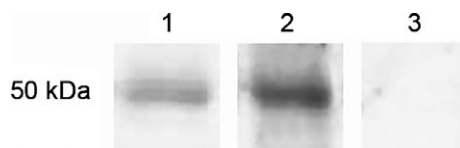

Fig 5. Western blot CYP17. Lane 1: immunoprecipitate. Lane 2: normal adrenal homogenate. Lane 3: negative control. At approximately 50 kDa, CYP17 bands are visible in lane 1 and lane 2, but not in the negative control. kDa = kilodalton, CYP17 = 17α-hydroxylase/17,20-lyase.

primates and rodents.[11,32] This specialization has resulted in a partial loss of function of one of the gene duplicates, since the ancestral CYP11B1 enzyme was able to catalyze 11β-hydroxylation, 18-hydroxylation, and 18-oxidation, while the primate and rodent CYP11B1 can only perform 11β-hydroxylation. Primate and rodent CYP11B2 is more similar to the ancestral enzyme than CYP11B1, as CYP11B2 can catalyze all 3 reactions (Fig 1).[6] The presence of multiple functional CYP11B enzymes and *CYP11B* pseudogenes in different species could indicate that duplication of the *CYP11B* gene occurred in a common ancestor mammal, and that loss of function, silencing, or partial deletion of 1 or multiple *CYP11B* genes occurred independently in the following evolution of the different species. Hypothetically, this might have occurred in canine spp. as well. The results of this study suggest that there is only 1 *CYP11B* gene present in the canine genome. Zone-dependent steroid synthesis must therefore depend on a different zone-specific enzyme.

Relative mRNA *CYP17* expression was significantly higher in the zF compared with the zG, which was confirmed by positive CYP17 IHC staining in zF and zR only. This is similar to human and guinea pig adrenals, in which CYP17 is expressed in the zF and zR, but not the zG.[35,36] Zone-specific expression of CYP17 is an important factor in canine zone-specific steroidogenesis, providing an explanation for the fact that cortisol synthesis is limited to the zF and zR. While rats and mice are widely used as models for human steroidogenesis, these animals do not possess CYP17 and therefore cannot produce cortisol. Ferrets do express CYP17, but their zonation pattern is different from that of humans.[37] The fact that canine adrenal zonation is similar to that of humans and dogs also express CYP17, and therefore have the same end product of the glucocorticoid pathway, indicates that dogs provide a better model for human steroidogenesis than rodents.

Since CYP11A1 and StAR are necessary for the first steps in steroidogenesis, ie, both aldosterone and cortisol production, the significantly higher relative expression of *StAR* and *CYP11A1* in the zF compared with the zG was not expected. However, although multiple studies in rats and humans have shown no difference in *StAR* expression between the zG and zF,[38–40] Peters et al.[41,42] describe that in nonstimulated rats (ie, not stimulated to produce extra aldosterone with a high-potassium diet), *StAR* mRNA expression is most prominent in the zF and zR, whereas the zG shows less *StAR* expression. Dogs used in this study received a regular diet with normal potassium content and were thus not stimulated to produce extra aldosterone. Higher expression of *StAR* in the zF than in the zG could be explained by the fact that the transport of cholesterol to the inner mitochondrial membrane, as facilitated by StAR, is the rate-limiting step in steroidogenesis.[43–45] *CYP11A1* has also been documented to play a rate-limiting role in steroidogenesis.[5,6] Higher expression of CYP11A1 and *StAR* in the zF than in the zG could ensure the greater production rates of glucocorticoids compared with mineralocorticoids in dogs.

Interestingly, bovine CYP11A1 has been documented to modulate CYP11B1 activities, while human CYP11A1 does not affect the product pattern of bovine
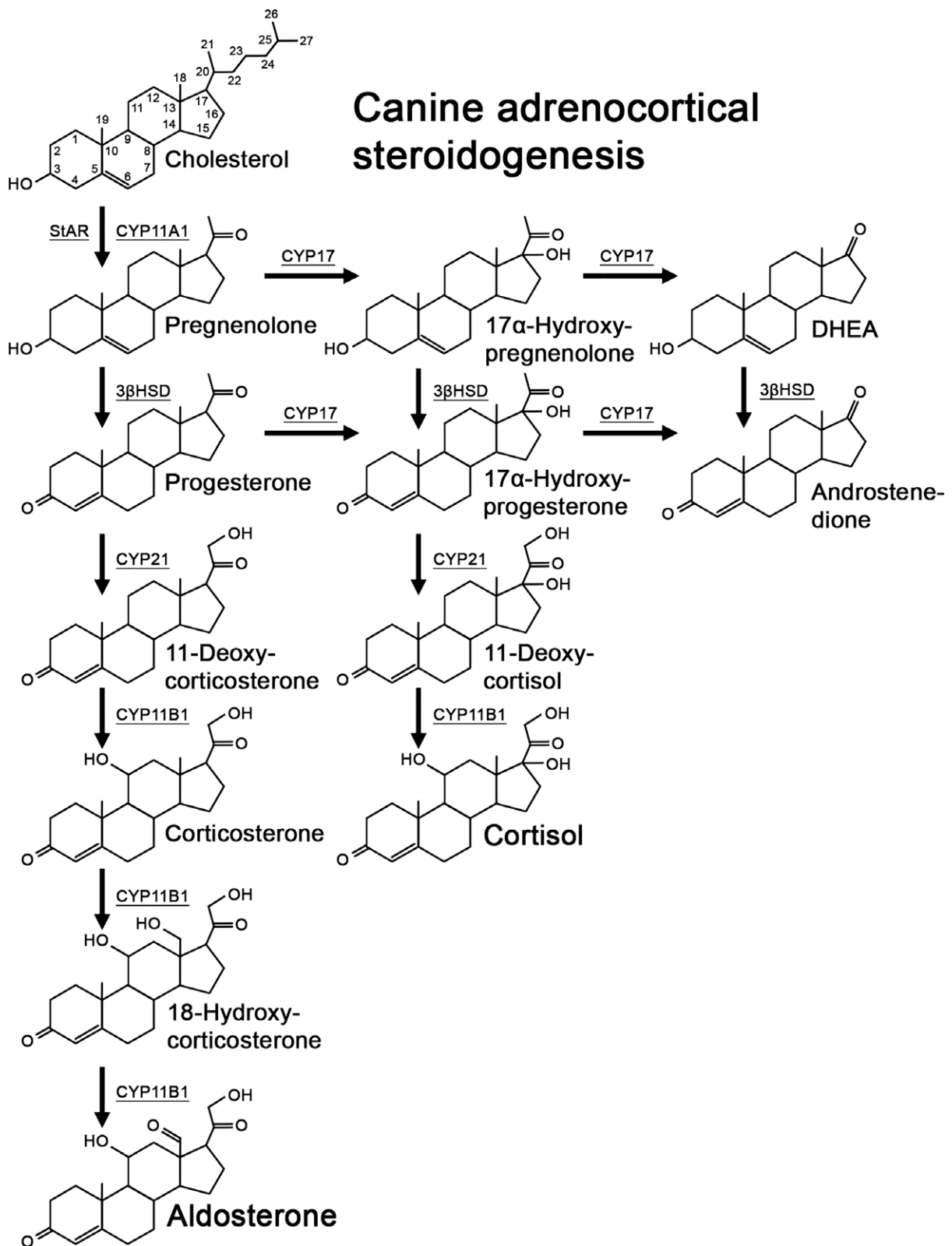
Fig 6. Proposed canine adrenocortical steroidogenesis. StAR = steroidogenic acute regulatory protein, CYP11A1 = cytochrome P450 side chain cleavage, 3βHSD = 3β-hydroxysteroid dehydrogenase, CYP17 = 17α-hydroxylase/17,20-lyase, CYP21 = 21-hydroxylase, CYP11B1 = 11β-hydroxylase cytochrome P450.

or human CYP11B1 or −2. Cao and Bernhardt found that cotransfection of bovine CYP11A1 with CYP11B1 resulted in an increase of 11β-hydroxylation and a decrease of 18-hydroxylation and 18-oxidation.[46,47] This could be due to a direct interaction of CYP11A1 with CYP11B1, which causes conformational changes in CYP11B1 that result in a different binding of the substrates and intermediates.[7,46] Possibly, in species which possess only 1 CYP11B enzyme, high CYP11A1 expression in the zF stimulates 11β-hydroxylation and inhibits 18-hydroxylation and 18-oxidation, while this inhibition is absent in the zG due to lower CYP11A1 expression.

CYP21 and 3βHSD are essential for both glucocorticoid and mineralocorticoid synthesis and have no rate-limiting functions.[1,48] The results of this study demonstrated no difference in expression between the zones, in agreement with the known literature.

ACTH is known to be responsible for glucocorticoid production in the zF,[49] but an acute rise in circulating ACTH concentration is also able to stimulate aldosterone secretion. This requires the presence of *MC2R* in the zG,[50,51] in concordance with the results of this study.

In humans, mice, and rats, SF-1 is known to be localized in all 3 layers of the adrenal cortex.[52] In humans, SF-1 is essential for full expression of, among others, MC2R, StAR, CYP11A1, 3βHSD, CYP17, CYP21, and CYP11B1, but not CYP11B2. The lack of CYP11B2 stimulation by SF-1 is most likely due to a different transcription mechanism.[53] A recent study from our group found immunohistochemical SF-1 staining throughout the entire normal canine adrenal cortex.[54] As expected, this study showed no difference in *SF-1* expression between the zF and zG in canine adrenals.

In conclusion, in this first study concerning canine steroidogenesis, we propose a canine steroidogenesis scheme (Fig 6) with only 1 CYP11B enzyme, which implies that the zone-specific production of aldosterone and cortisol is probably due to zone-specific CYP17 expression. CYP17 expression in the zF is crucial for cortisol synthesis, while lack of CYP17 in the zG restricts steroidogenesis to mineralocorticoid production. The zone-specific presence of CYP17 makes it an attractive target for selective inhibition of cortisol synthesis without affecting mineralocorticoid production in the zG.

Footnote

Acknowledgments

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