Thyrotropin releasing hormone receptor and the adrenal gland

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Abstract

Thyrotropin releasing hormone (TRH) is a hormone which is made in the hypothalamus and attaches in the pituitary gland to the thyrotropin releasing hormone receptor (TRHR). By binding it stimulates the secretion of TSH which stimulates the secretion of T4 and T3. In horses with Cushing disease it also stimulates the ACTH secretion and subsequently the cortisol secretion. This fact is used in the TRH stimulation test to diagnose the horses with Cushing. In dogs TRH administration has a different result. After administrating TRH the cortisol concentration rises without an increasing in the ACTH concentration, in both healthy and Cushing dogs. These results can be explained when there would be expression of *TRHR* in the adrenal gland. This is the object of this research.
In six adrenal gland samples and two pituitary gland samples the expression of *TRHR* is examined. After RNA isolation and cDNA synthesis, PCR is performed. In several samples of the adrenal glands and in both pituitary gland samples the *TRHR* is found. To evaluate the quantity of the *TRHR* in the adrenal gland, qPCR is used. Several gradients are performed but all did not succeed due to the lack of purity and quality of the products. Therefore no information about the quantity of the *TRHR* is available.
Immunohistochemistry of the eight different adrenal gland sections revealed the presence of the receptor in the cortex and medulla of the adrenal glands. More research has to be done to measure the exact amount of the *TRHR* in the adrenal gland, to evaluate the difference between male and female, between cortex and medulla and between the different zones of the cortex. Also the whole receptor should be sequenced to examine the variation between different dogs and to compare the *TRHR* with the canine *TRHR* gene.

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Introduction and aim of the study

The thyrotropin releasing hormone receptor (TRHR) is expressed in the pituitary gland. Thyrotropin-releasing hormone (TRH) is a hormone which is made in the hypothalamus and attaches to the TRHR in the pituitary gland. By this binding it normally stimulates secretion of thyroid stimulating hormone (TSH). This hormone is secreted and transported to the thyroids. In the thyroids it stimulates the production and secretion of T4 and T3 (figure 01). T4 and T3 have a negative feedback mechanism to the hypothalamus and the pituitary gland to prevent an overproduction (1).

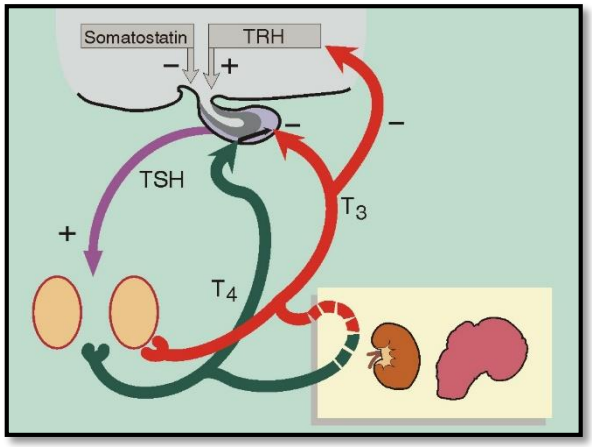


Figure 01 Hypothalamus-pituitary-thyroid axis
H.S. Kooistra

In horses with pituitary pars intermedia dysfunction (PPID) or Cushing syndrome, which is caused by an adenoma of the pars intermedia of the pituitary gland, TRH is also involved in another mechanism. Hereby the TRH stimulation test, can be used to diagnose horses with Cushing syndrome. After performing the TRH stimulation test, in horses with Cushing, the ACTH secretion is stimulated and the concentration rises. ACTH stimulates the adrenal glands to produce and secrete cortisol. Subsequently due to the rise of ACTH also the cortisol concentration increases. This in contrast to the healthy horses in which the ACTH concentration remains stable after administrating TRH (02, 03, 04). Typical clinical signs of Cushing in horses are long hair, weight loss, long shedding pattern, polydipsia and polyuria, abnormal sweating and laminitis (5) (figure 02).

Figure 02 Horse showing clinical signs of Cushing syndrome
H.S. Kooistra

Cushing syndrome is also rather common in dogs due to a pituitary gland tumor. The major part (80-85%) of the dogs with Cushing syndrome suffer from a pituitary adenoma which give the pituitary dependent hypercortisolism. These adenomas are predominantly found in the anterior lobe of the pituitary gland (6, 7). Administration of TRH to dogs with Cushing syndrome did not result in a significant rise in ACTH. Surprisingly, it only results in a significant increase in plasma cortisol secretion. Even in healthy dogs (8). These results can be explained when there would be expression of *TRHR* in the adrenal gland. This is the object of this research. Dogs have other clinical signs compared with horses. Dogs show different clinical signs compared with horses. Polyuria, polyphagia, centripetal obesity and atrophy of the muscles and skin are dominating features in dogs with Cushing (7) (figure 03).

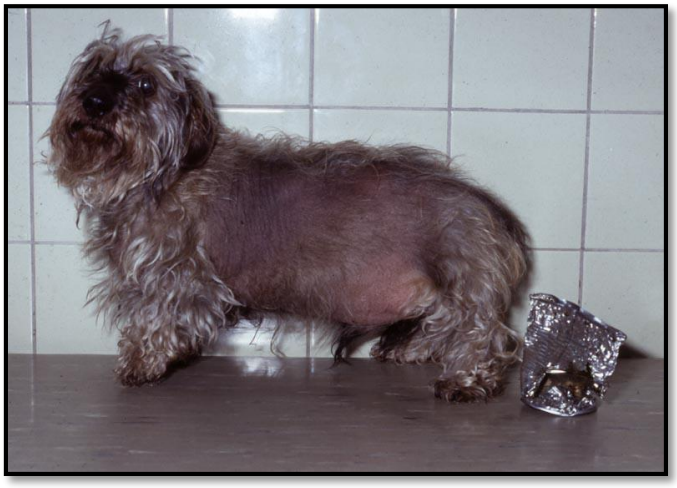


Figure 03 Dog showing clinical signs of Cushing syndrome
H.S. Kooistra

The aim of this research is to evaluate the expression of the *TRHR* in the canine adrenal gland. To measure the expression of the canine *TRHR*, the RNA from adrenal gland tissue will be isolated. With this RNA cDNA will be made which can be used to detect the presence of *TRHR* by PCR. Specific primers will be designed and used for PCR and qPCR. Also immunohistochemistry will be done to evaluate the localization of the TRHR in the adrenal gland.

To examine the adrenal glands, the anatomy of the glands had to be known. The adrenal glands are positioned in front of the kidneys and can be divided in two different parts, the inner medulla and the outer cortex. The cortex can further be divided in three different zones. From inner to outer, the zona reticularis, fasiculata and the glomerulosa (figure 04). All these different parts have different functions. The medulla produces and secretes epinephrine and norepinephrine, which are catecholamines. The catecholamines are for example responsible for stimulation of glycogenolysis, lipolysis and increasing in metabolic rate, alertness and rate of myocardial contraction. The zona reticularis and fasiculata produce glucocorticoids such as cortisol. The glucocorticoids secretion is influenced by ACTH from the anterior pituitary. The zona glomerulosa produces and secretes mineralocorticoids such as aldosterone which has an important function in the water and salt regulation (9).

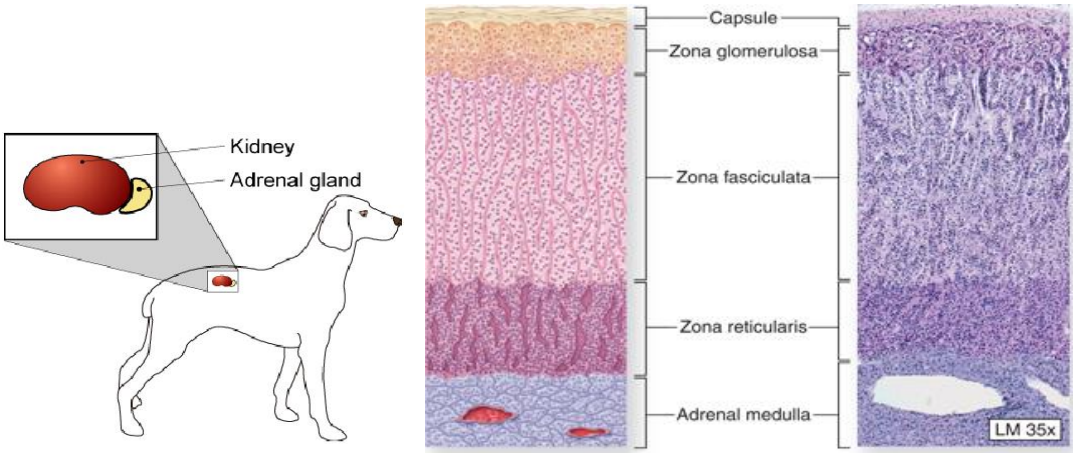


Figure 04 Anatomy of the adrenal gland
VCA Animal Hospitals and BPCC

Hypotheses

"The *TRHR* gene is not only expressed in the canine pituitary gland but also expressed in the canine adrenal gland."
"The *TRHR* in the canine adrenal gland is the same as in the canine pituitary gland and is well distributed in the adrenal gland."

Materials and methods

Tissues

Canine adrenal gland tissue
Approximately 30 milligrams of six canine adrenal glands of healthy dogs is used. The adrenals are taken from euthanized healthy dogs between December 2013 and September 2014. (table 01 for detailed information). The tissues are in the meantime stored at -70°C.

Table 01: Origin of the adrenal gland samples*

Sample number	Description	Date of collection	Gender	Date of birth	Patient number
1	Normal adrenal cortex	10-09-2014	Unknown	-	-
2	Normal adrenal gland	25-02-2014	Male	19-04-2009	7207859
3	Normal adrenal cortex	02-12-2013	Male	14-08-2012	909041
4	Normal adrenal gland	16-04-2014	Female	25-05-2012	898503
5	Normal adrenal cortex	15-09-2014	Female	2012	-
6	Normal adrenal gland	04-03-2014	Male	25-09-2007	7140151

**Due to sudden available samples due to sudden deaths not everything is known.*

Pituitary gland tissue
cDNA of several pituitary glands of healthy dogs is already available. Two of these samples are used in this research as positive controls. The samples are already 10x diluted. By this fact no pituitary gland tissue is needed.

Pituitary gland sections and formalin fixated tissue
Several sections of a pituitary gland of a healthy dog are used. Also a part of the pituitary gland which was already fixed in formalin is available, to provide more sections when these are needed.

Adrenal gland sections
In total eight different formalin fixated adrenal glands of healthy dogs are used to make several sections for immunohistochemistry.

Techniques

RNA isolation, DNA digestion and RNA clean-up
RNA is isolated by using the RNeasy® Mini Kit (Qiagen®) with manufacturer's protocol. In this kit, the selective binding properties of a silica-based membrane is combined with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane. The frozen tissue (approximately 30 mg) is disrupted in Buffer RLT and homogenized with a mortar. The disruption is necessary to release all the RNA contained in the samples. Incomplete disruption results in significantly reduced RNA yields. Homogenization is necessary to reduce the viscosity of the lysates produced by disruption. DNA removal is necessary for the RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundance target). Residual DNA is removed by a DNase digestion after RNA purification. The DNA digestion is performed to prevent DNA interference with the reactions later on. RNA has undergone a final clean-up to remove all the chemicals used during the methods to isolate RNA (10). The weight of the tissue samples is estimated, not weighted, around 30 milligram by cutting a little piece of the frozen adrenal glands. After isolation all the RNA samples are stored at a temperature of -28°C. The RNA amount of all the samples is measured using the Nanodrop®, see column two of table 10.

cDNA synthesis
To produce cDNA out of the RNA of the canine adrenal glands the iScript™cDNA Synthesis Kit® (BioRad®) is used with manufacturer's protocol. The iScript reverse transcriptase is RNAse H+ and pre-blended with RNAse inhibitor. The specific blend of oligo (dT) and random hexamer primers in the iScript Reaction Mix works with a wide variety of targets and thereby also suitable for this reaction. The kit provides a good solution for a two-step PCR. After synthesis also the samples are stored at a temperature of -28°C.
According to the protocol, the used RNA template should not be more than 1µg RNA, since this is the maximum the iScript™cDNA Synthesis Kit can process. This maximum amount of RNA is used to produce the cDNA (11). In table 02 the amounts of RNA template, RNase free water, reaction mix and reverse transcriptase are summarized.

Table 02: Reaction components used in the 20µl reaction to produce cDNA

Sample	Reaction mix (µl)	Reverse transcriptase (µl)	RNA template (µl)	RNase free water (µl)
1	4	1	4,77	10,23
2	4	1	10,94	4,06
3	4	1	3,02	11,98
4	4	1	4,96	10,04
5	4	1	4,66	10,34
6	4	1	6,55	8,45

Primers

To multiply only the region of the expected *TRHR* a specific primer set is designed. This set is designed based on the criteria for primers used in the qPCR technique. Thereby the primers can be used for PCR and qPCR and generate a short sequence of less than 200 bp. In this case a fragment of approximately 112 bp indicative for the *TRHR* region will be the product.
The designed primer set:

- Forward primer: 5' TAGATGTTTCAACAGCACAG 3' -> forward primer 1
- Reverse primer: 5' TGACAACCACAAGAGTCC 3' -> reverse primer 1

This primer set is called set 1.

To multiply a longer region of the expected *TRHR* region another primer set is designed. The primers are chosen out of a list of different primers which are already available in the lab. The chosen set of primers will give a product of approximately 636 bp of the *TRHR* region.
The designed primer set:

- Forward primer: 5’ GCTCTGGTTCTTCTTGCTGG 3’ -> forward primer long
- Reverse primer: 5’ GATGTCATCAAGCTCTGTGC 3’ -> reverse primer long

This set is called set long.

To perform qPCR several new primer sets are designed. The different primer sets are as well combined with each other to create more options to test and to find the optimal set. The designed primer sets are displayed below.
Set 2, which will give a product of 113 bases (1155-1268), indicative for the *TRHR* region:

- Forward primer 2: 5' AGTATCTTCAAGGAAGCAGGT 3'
- Reverse primer 2: 5' GGGCTGGTGAGAAATGAG 3'

Set 3, which will give a product of 143 bases (1041-1184), indicative for the *TRHR* region:

- Forward primer 3: 5' CTTAAATCCCATTCCTTCAGATCC 3'
- Reverse primer 3: 5' ATCTTGGTGACCTGCTTCC 3'

Set 4, which will give a product of 132 bases (1147-1279), indicative for the *TRHR* region:

- Forward primer 4: 5' AACAGCACAGTATCTTCAAGGA 3'
- Reverse primer 4: 5' TTTCTTGGAAAGGGCTGGT 3'

Set 5, which will give a product of 99 bases (1163-1262), indicative for the *TRHR* region:

- Forward primer 5: 5' CAAGGAAGCAGGTCACCA 3'
- Reverse primer 5: 5' GTGAGAAATGAGTTGACAACCA 3'

Also different combinations of primers are used during this research.

PCR

PCR is done to multiply the cDNA fragments. For each sample a solution of 15µl and the standard protocol of the lab for PCR is used. The composition of the mixture is shown in table 03. All the six canine adrenal gland samples and the two canine pituitary gland samples are used with this method. The different steps of the temperature are shown in table 04.

Table 03: Standard protocol for PCR used for the six canine adrenal glands and the two canine pituitary gland samples

Components	**For 1 reaction (µl)**	**Concentration**	**10x mix****
10x PCR buffer	1,5	1x	15
$MgCl_2$ (50 mM)	0,6	2 mM	6,0
dNTP's (10 mM)	0,3	200 µM = 0,2 mM	3,0
Primer F (10 µM)	0,75	0,5 µM	7,5
Primer R (10 µM)	0,75	0,5 µM	7,5
Platinum Taq	0,15	-	1,5
cDNA (25 ng/µl)*	2	25 ng	-
mQ (RNase free water)	8,95	-	89,5

**Standard is to put 2 µl of the cDNA sample in the mix, to ensure that the minimum amount of 25 ng is reached. The cDNA is for every sample different and therefore not mixed in the mix.*

***In total 8 samples have to be multiplied by the PCR procedure. Therefor a mix for 10 samples is made, to ensure there is enough for all the samples.*

Table 04: Standard PCR protocol for temperature, time and cycles.

Temperature	**Time**	**Cycles**
95°C	5 min	1
95°C	30 sec	35
55°C	30 sec	35
72°C	30 sec	35
72°C	10 min	1
20°C	Endless	1

Optimization of the PCR

To have a one band result on electrophoresis after PCR with primer set long an ideal protocol has to be found. To establish this, the temperature is increased, with 3°C, each time. See table 05 for the two tested protocols.

Table 05: Protocols used to optimize the PCR

Temperature first run	**Temperature second run**	**Time**	**Cycles**
95°C	95°C	5 min	
95°C	95°C	30 sec	35
55°C	58°C	30 sec	35
72°C	72°C	30 sec	35
72°C	72°C	10 min	
20°C	20°C	Endless	

Electrophoresis

After PCR agarose gel electrophoresis is performed. The standard protocol of the lab is used. The expected length of the products from the first experiment is 112 bp. Because

of this short length of the cDNA fragments an agarose gel of 2% is used to run the electrophoresis. The cDNA fragments used for the second experiment are about four times as large as the fragments of the first experiment. Therefore the agarose gel has a concentration of 1,5% instead of 2%. The same 100 bp size standard ladder is used to compare the length of the bands.

Gel extraction

To ensure the purity of the products, after electrophoresis of the PCR fragments, gel extraction is performed. Extraction is only used for two of the adrenal gland samples which showed the clearest bands on the electrophoresis gel. The control samples of the pituitary gland are very clear, so gel extraction of these samples is not necessary. Gel extraction is performed by using the QIAquick® Gel Extraction Kit (Qiagen®), with manufacturer's protocol. The Kit contains a silica membrane assembly for binding of DNA in high-salt buffer and elution with low-salt buffer or water. The purification procedure removes primers, nucleotides, enzymes, mineral oil, salts, agarose, ethidium bromide, and other impurities from the DNA samples (12).

Exo 1

If necessary, to degrade the previous used forward and reverse primers, the Exo 1 digestion is performed. The standard protocol of the lab is used by adding to 10-15µl PCR product an amount of 1µl Exo1 (2U/µl). The amount is mixed by pipetting and the EXO 1 program is used according to table 06.

Table 06: Program used in the Exo 1 digestion reaction

Degree (°C)	**Time (min)**
37°C	45 min
75°C	15 min
4°C	Endless

Tercycle

The standard Tercycle protocol of the lab is used for the control samples (C1 and C2). This protocol is also used during the second experiment with primer set long. The used components for this reaction are shown in table 07. The products derived by the gel extraction are much more diluted compared with the control samples which did not undergo the gel extraction. Therefore these samples have a different protocol. In the Tercycle, of the gel extraction samples, a reaction in 20µl is used with another composition (table 08). For all Tercycles the same program is used (table 09).

Table 07: Standard protocol for the Tercycle of a 10µl reaction

Fluid	**For 1 reaction (µl)**	**Concentration**
Purified PCR product	2*	
Betaine 4M	2,5	1M
BDT	1	
Primer (3,2 µM)	1	320nM
5x sequence buffer	2	1x
mQ	1,5	

**Of sample C2 only 2µl was available. Two samples of 2µl are needed so the sample of C2 is diluted with 2µl mQ to achieve two samples of 2µl.*

Table 08: Protocol used for the Tercycle of a 20µl reaction of the adrenal gland samples

Components	**For 1 reaction (µl)**
Purified PCR product	11,5
Betaine 4M	2,5
BDT	1
Primer (3,2 µM)	1
5x sequence buffer	4
mQ	-

Table 09: Standard protocol for temperature, time and cycles of the Tercycle.

Temperature	**Time**	**Cycles**
96°C	5 min	
96°C	30 sec	35 cycles
50°C/55°C*	15 sec	35 cycles
60°C	30 sec	35 cycles
4°C	Endless	

** 50°C or 55°C. Depends on the previous PCR. Did the PCR occur with 55°C, the Tercycle has to be done at 50°C. Has the PCR run at a temperature above the 55°C, the Tercycle has to be done at a temperature of 55°C. Different temperatures are used for PCR, so both protocols for the Tercycle are used during this research.*

cDNA sequence analysis and comparison with the canine (canis lupus familiaris) genome

Sequencing is done by using the ABI3130XL® protocol available in the lab. To see if the sequence of the produced fragments is similar to the expected *TRHR* region of the canine genome, the sequence is compared with the whole canine genome at the Ensembl website by performing a BLASTN analysis (13).

qPCR

To multiply and quantify the products, qPCR is performed according to the iQSYBRgreensuperMix® protocol summarized by the lab. All the samples (sample 1-6 of the adrenal gland and C1 and C2 of the pituitary gland) are used in the reactions. RPL13, RPS19, SDHA, GAPDH and HPRT are used as reference genes. To evaluate the right temperature for the specific primers to run the qPCR, several gradient qPCR's are performed with a gradient of 55°C-65°C.
Primer set 1-5 are used in these gradients. Different combinations are made with the forward and reverse primers:

- Forward 1 + reverse 2
- Forward 2 + reverse 1
- Forward 2 + reverse 2
- Forward 3 + reverse 3
- Forward 4 + reverse 4
- Forward 5 + reverse 5
- Forward 3 + reverse 5
- Forward 4 + reverse 3
- Forward 5 + reverse 4

Immunohistochemistry of the adrenal gland and pituitary gland samples

The used antibody for immunohistochemistry is Anti-TRH Receptor antibody (ab72179) produced by Abcam®, which is a specific *TRHR* antibody. It is rabbit polyclonal and

directed to the TRH Receptor and suitable for immunohistochemistry. According to the manufacturer, the antibody reacts with canine, human, chimpanzee and monkey (14).

To perform an optimal immunohistochemistry reaction, several protocols are tested to find the most suitable. First the pituitary gland sections are used to evaluate the optimal protocol, medium and dilution to expose the target epitope for this antibody. Pituitary gland sections are chosen due to the known presence of the *TRHR* (15, 16). Whereas the manufacturer of the antibody had no protocol for the *TRHR* antibodies, to start one of the lab protocols is used. Citrate and TE, for ten minutes at 98°C and just only the TBS 0,025% Triton buffer at room temperatures, are evaluated first as medium and buffer. Thereafter several pituitary gland and adrenal gland sections with different concentrations of the TRHR antibody with both the TE medium and citrate medium are evaluated. Eventually ten minutes in the TE medium at 98°C, with 1:50 dilution of the *TRHR* antibody and buffer TBS with 0,025% Triton and 0,1% Tween is the best to evaluate the TRHR in the adrenal gland. Several adrenal gland sections are stained using these facts to evaluate the presents of TRHR in the adrenal gland. Appendix 03 shows the final protocol.

Results

RNA isolation, DNA digestion and RNA cleanup

After RNA isolation, DNA digestion and RNA cleanup according to manufacturer's protocol, the collected amount of RNA, the 260/280 ratio and the 260/230 ratio are measured by the Nanodrop®. The results are shown in table 10. The 260/280 ratio is a measure for the purity of the RNA. A ratio of approximately 2,0 is accepted as 'pure' for RNA. A lower ratio indicates the presence of contamination by proteins or phenol. The 260/230 ratio is a secondary measure of nucleic acid purity. A low ratio may indicate co-purified contaminants. Each sample has an amount of 50µl. The total of RNA per sample is calculated and also shown in table 10. The amount of sample 1, 4 and 5 are very similar. Sample 2 has clearly the lowest amount and sample 3 by far the most.

Table 10: The isolated RNA samples.

Sample number	RNA (ng/µl)	260/280*	260/230**	Remarks	Total RNA (µg)***
1	209,5	2,05	1,33		10,475
2	91,4	2,04	0,81	Least amount	4,57
3	331,5	2,02	1,26	Most amount	16,575
4	201,5	2,02	1,66		10,075
5	214,7	2,06	1,06		10,735
6	152,6	2,03	1,79		7,63

**Is a measure for the purity of the RNA.*

*** Is a measure of nucleic acid purity.*

****Measured RNA ng/µl x 50 : 1000 = total RNA (µg)*

PCR to multiply a *TRHR* fragment

After RNA isolation, cDNA synthesis is performed. No more than 1µg RNA can be used in the iScript™cDNA Synthesis Kit to synthesize the cDNA. After making the cDNA the fragments are multiplied by PCR and made visible with electrophoresis. The first PCR is performed with the six adrenal gland samples and the two pituitary gland samples with primer set 1. After performing the PCR, electrophoresis with an agarose gel of 2% is used to make the products visible (figure 05). The first lane in the figure represent the 100 bp ladder. Lanes 2-7 represent the adrenal gland samples and the eighth and ninth lane represent the pituitary gland samples, which function as positive controls. The expected *TRHR* region has 112 bp. So the bands of the eight products are expected between the 100 bp and 200 bp band of the 100 bp ladder. In lanes 2-7 several bands are visible at different heights. In lane 2 several bands (one really high, just below the arrow, and several a bit further down) are visible. From the adrenal gland samples, the bands in lane 5 and 6 are the most clear. In lane 8 and 9 one clear band is visible at the expected height of the *TRHR* fragment. Also in several lanes of the adrenal gland samples several bands are visible at the expected height of the *TRHR*.

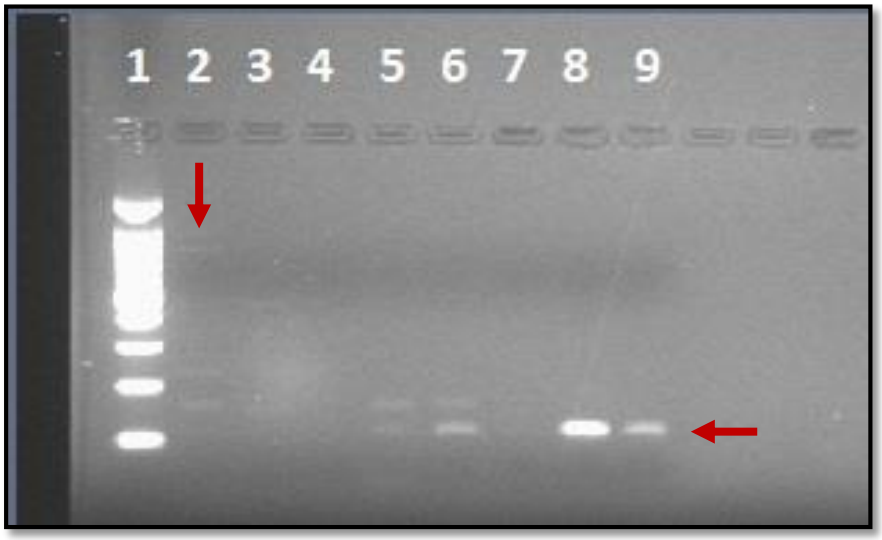


Figure 05 Expression of the expected TRHR region in the adrenal gland and the pituitary gland.

Lane 1: the 100bp ladder, 2-7: adrenal glands (1-6), 8-9: pituitary glands (C1 and C2).

Purification, DNA sequencing and comparison with the canine genome

To ensure the identity of the product of the adrenal gland samples, gel extraction is performed. Only samples 4 and 5 are used, since these have the clearest bands at the expected height of the *TRHR* after electrophoresis. These two samples together with the two pituitary gland samples are analyzed and sequenced to evaluate whether the bands of figure 05 represent the fragment of the *TRHR*. Due to an error, the program is not able to show the sequence after analyzing the fragments. Therefore a short fragment of sample 5 of the adrenal glands and sample 2 of the pituitary glands are sequenced by visual inspection of the raw data and compared by BLASTN at the ensembl site to determine if the sequence belongs to the fragment of the *TRHR* region.

From sample 5, the DNA sequence with a length of 37 bases is compared with the canine genome. From the pituitary gland a DNA sequence with a length of 50 bases is compared with the canine genome. A short fragment of the analyses is shown in figures 06 and 07. The displayed sequences are 100% similar to each other. Both fragments shown in the figures begin with a green top followed by two red tops and so one. Not the whole fragment of 112 expected bases could be sequenced caused by the presence of background. After comparison of the samples with the canine genome both DNA sequences belongs most likely to the *TRHR* region and had a similarity of 100% with the canine genome. The fragment of sample B5 represent the *TRHR* region with an E-value of $3{,}0x10^{-6}$. The fragment of C2 represent the *TRHR* region with an E-value of $8{,}0x10^{-20}$. The detailed alignment of the samples with the canine genome is also evaluated and shown in figures 08 and 09. No differences are observed between the obtained and expected DNA sequences.

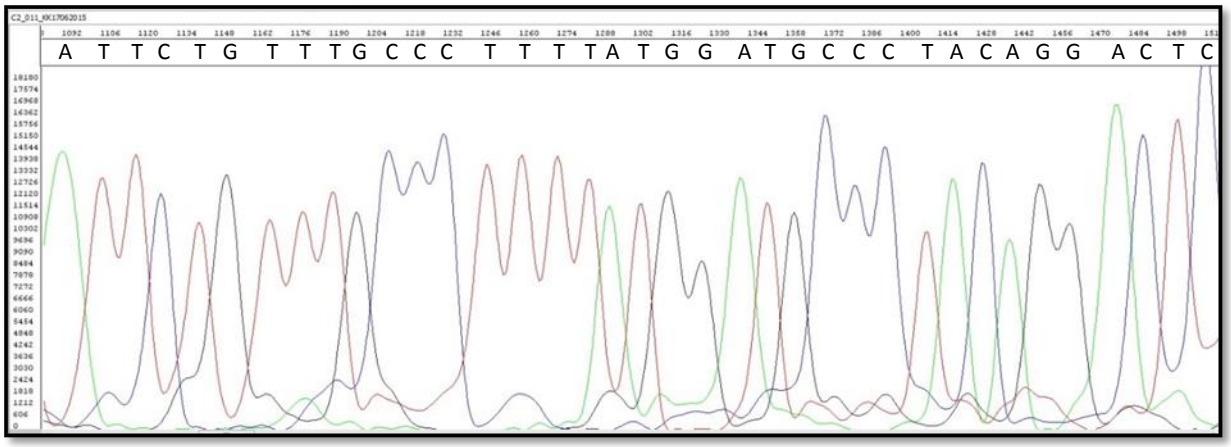


Figure 06: A fragment of the electrogram of sample C2 of the pituitary glands with forward primer 1.

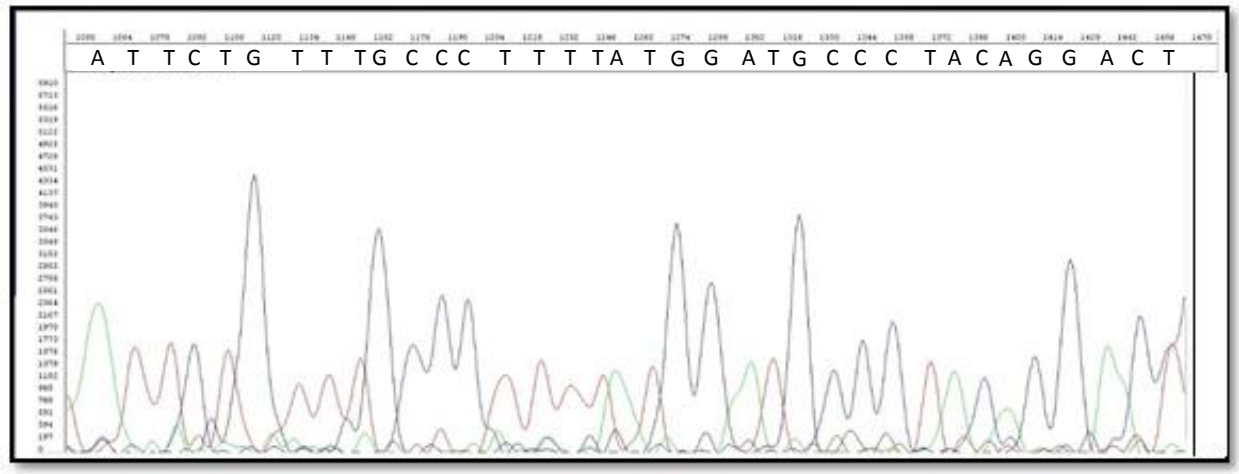
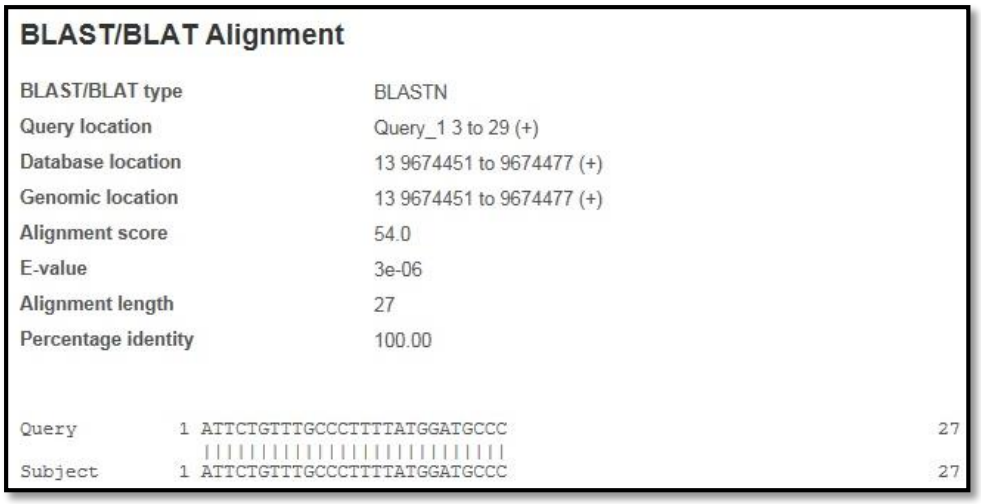


Figure 07: A fragment of the electrogram of sample B5 of the canine adrenal glands with forward primer 1.

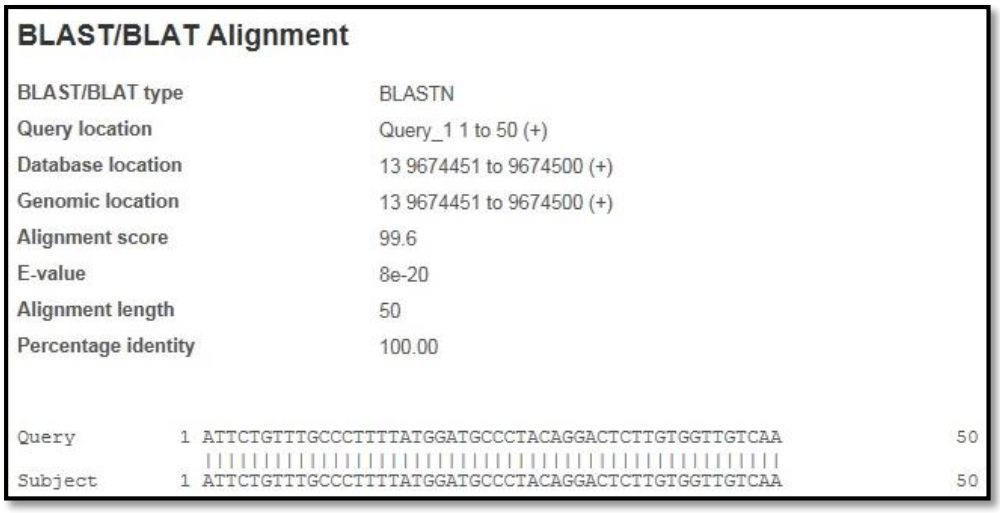


BLAST/BLAT Alignment

BLAST/BLAT type	BLASTN
Query location	Query_1 3 to 29 (+)
Database location	13 9674451 to 9674477 (+)
Genomic location	13 9674451 to 9674477 (+)
Alignment score	54.0
E-value	3e-06
Alignment length	27
Percentage identity	100.00

```
Query       1 ATTCTGTTTGCCCTTTTATGGATGCCC 27
              ||||||||||||||||||||||||||
Subject     1 ATTCTGTTTGCCCTTTTATGGATGCCC 27
```

Figure 08: Alignment of sample B5 of the adrenal glands (with forward primer 1) with the canine genome.



BLAST/BLAT Alignment

BLAST/BLAT type	BLASTN
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Database location	13 9674451 to 9674500 (+)
Genomic location	13 9674451 to 9674500 (+)
Alignment score	99.6
E-value	8e-20
Alignment length	50
Percentage identity	100.00

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Query       1 ATTCTGTTTGCCCTTTTATGGATGCCCTACAGGACTCTTGTGGTTGTCAA 50
              ||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     1 ATTCTGTTTGCCCTTTTATGGATGCCCTACAGGACTCTTGTGGTTGTCAA 50
```

Figure 09: Alignment of sample C2 of the pituitary glands (with forward primer 1) with the canine genome.

To evaluate a large part of the *TRHR*, a set of two other primers with the same adrenal and pituitary gland cDNA samples is used to perform PCR and electrophoresis. The length of this *TRHR* fragment is 636 bases and is expected between the 600 bp and 700 bp of the 100 bp ladder. After the first PCR and electrophoresis the result is not good enough, through the presence of a lot of bands (figure 10). In the figure the first lane represent the 100 bp ladder which is not clearly visible, in lane two and three no clear and separate bands are visible neither. Through this result a second PCR is performed, with a temperature of 58°C instead of 55°C (figure 11). In this figure the 100 bp is clearly visible. There is one band visible in the pituitary gland lane (lane 3) and also a light band at the same height is visible in the adrenal gland lane (lane 2). After the electrophoresis also these samples are analyzed and sequenced. Since the background is much more prevalent in the adrenal gland sample compared with the pituitary sample the quality of the adrenal gland sample is less. This is shown through the color of the bases. Blue is a good quality and with yellow the quality is less (figure 12 and 13). However the quality of the adrenal gland sample is less, it is no problem for the software to read the DNA and analyze the sequence the sample. To estimate the found sequence

is the sequence which represent the *TRHR* region, it is compared with the canine genome by BLASTN of the sequence against the canine genome at the Ensembl site (13). For the comparison, sample B5 of the adrenal glands and sample C2 of the pituitary glands are used. Both with the forward and the reverse primer of primer set long. The sequence of B5F (sample 5 of the adrenal glands with the forward primer) gave 47 hits, B5R (with the reverse primer) gave 19 hits, C2F (sample 2 of the pituitary glands with the forward primer) gave 80 hits and C2R (with the reverse primer) gave 29 hits. In all the cases the *TRHR* is the most likely region. The alignment and E-value of the samples are summarized in table 11. More details about the alignment of sample B5 and sample C2 with the forward primer are shown in figures 14 and 15.

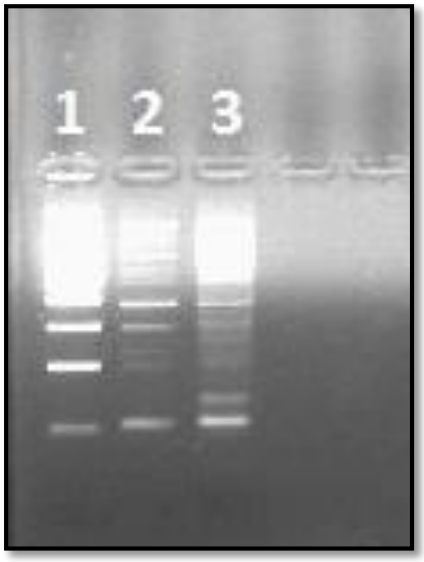

Figure 10: Electrophoresis result with primer set long, at a temperature of 55°C
**1: the 100bp ladder, 2: sample B5 of the adrenal glands, 3: sample C2 of the pituitary glands.*

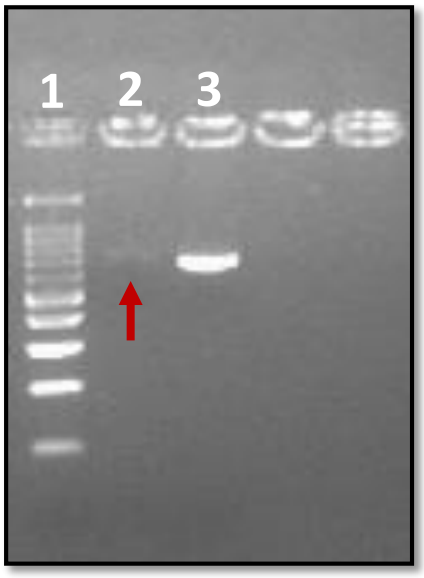

Figure 11: Electrophoresis result with primer set long, at a temperature of 58°C
** 1: 100bp ladder, 2: sample B5 (adrenal gland 5), 3: sample C2 (pituitary gland 2)*
*** Arrow: a light band is also visible in the adrenal gland lane*

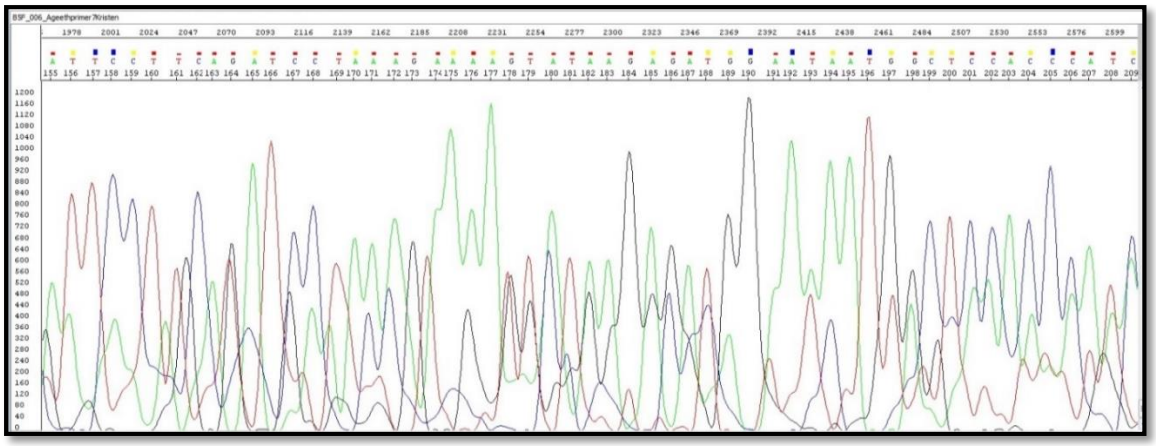

Figure 12: A fragment of the electrogram of sample B5 of the adrenal gland with forward primer long.

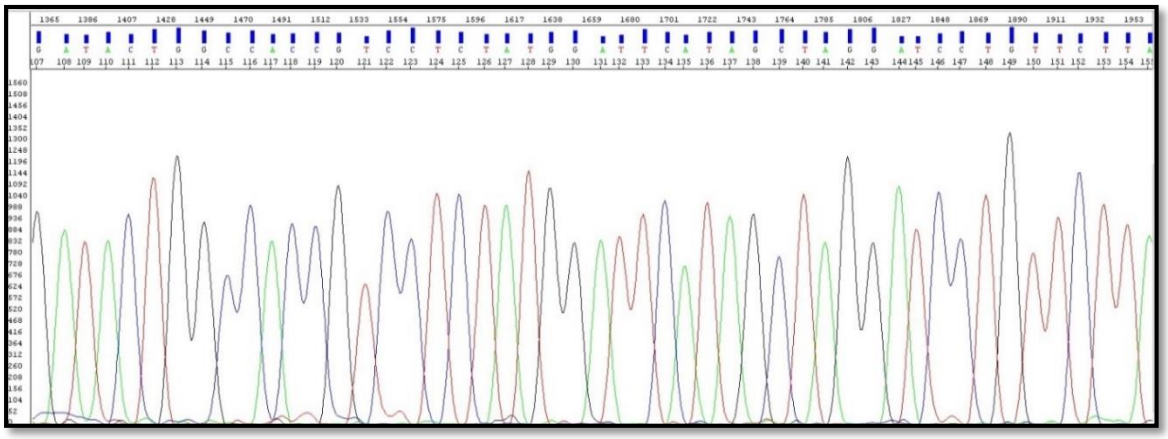

Figure 13: A fragment of the electrogram of sample C2 of the pituitary glands with forward primer long.

Table 11: Comparison of the expected TRHR region with the canine genome

Sample	Length	E-value	Alignment
5 of the adrenal gland (B5F), with forward primer	257 bases	$1{,}0x10^{-105}$	91,05%
5 of the adrenal gland (B5R), with reverse primer	112 bases	$4{,}0x10^{-28}$	91,07%
2 of the pituitary gland (C2F), with forward primer	278 bases	$9{,}0x10^{-142}$	97,84%
2 of the pituitary gland (C2R), with reverse primer	320 bases	$1{,}0x10^{-173}$	99,06%

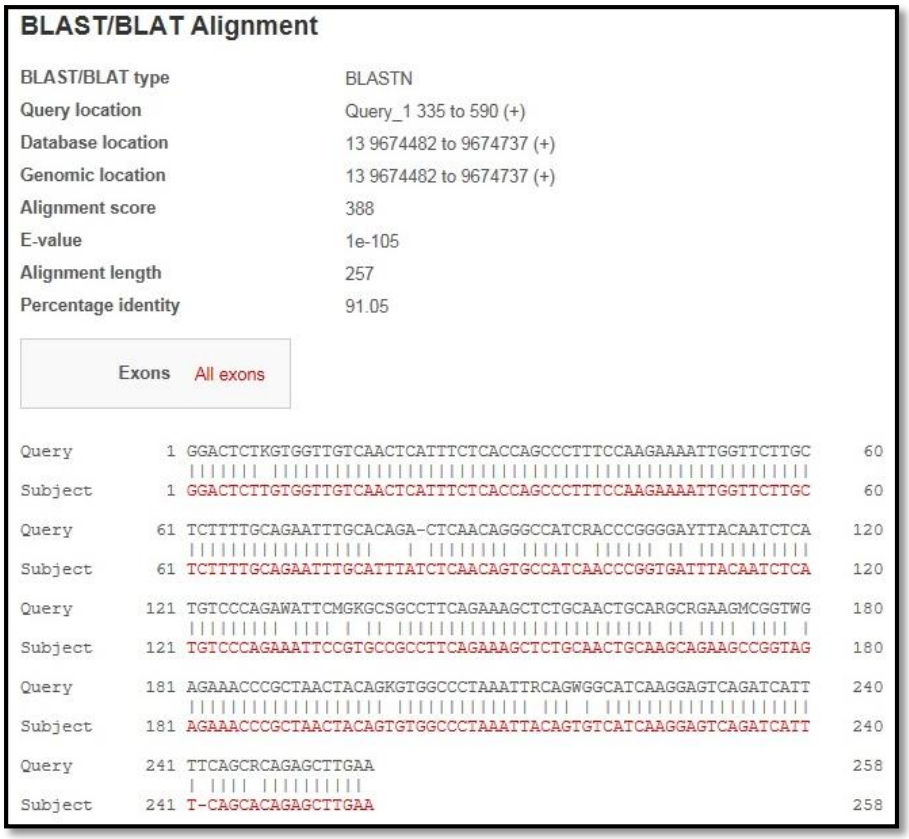

Figure 14: Alignment of sample B5 of the adrenal glands (with forward primer long) with the canine genome.

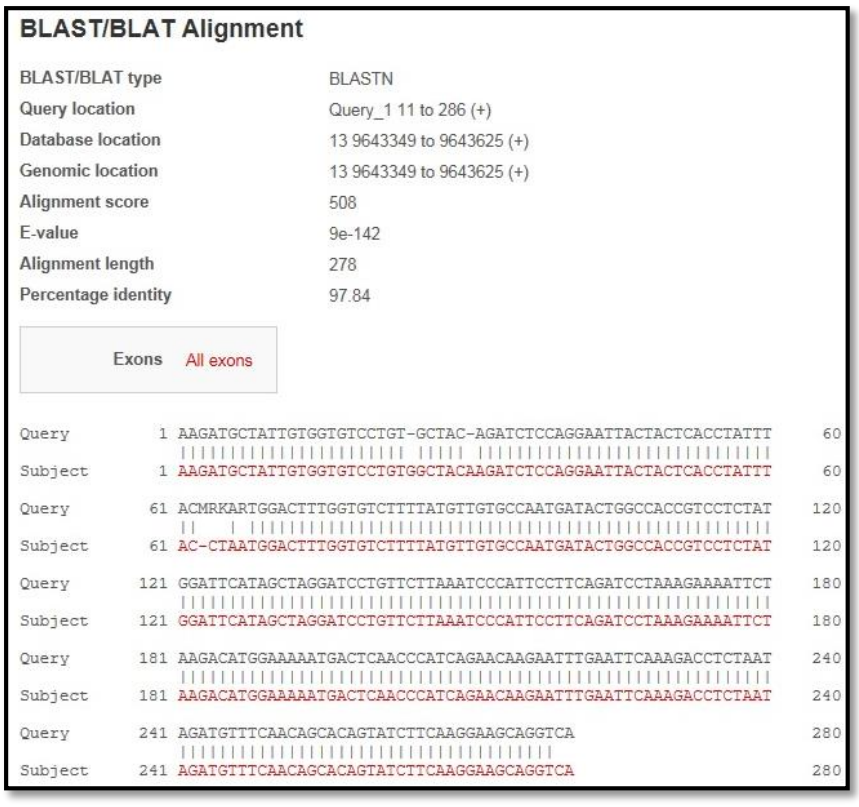

Figure 15: Alignment of sample 2 of the pituitary glands (with forward primer long) with the canine genome.

qPCR of the reference genes and the *TRHR*

First following protocol qPCR of the reference genes is performed to evaluate the adrenal and pituitary gland samples. Reference genes RPL13, RPS19, SDHA, HPRT and GAPDH are used. The thresholds are summarized in appendix 01. The melt curve, melt peak, amplification chart, E-value and r^2 from each reference gene are also summarized in appendix 01. All the reference genes have a good efficiency, between 101,1-102,8%, with an appropriate r^2.

After the qPCR of the reference genes, several gradients are performed to evaluate the proper temperature for the *TRHR* primer combinations to run the qPCR of the samples. The gradients are performed with combinations of primer set 1-5. Only the melt peaks are displayed in appendix 02 to illustrate the failed purity of the derived products, which is necessary to perform a qPCR. The results of all the combinations are not good enough to perform a qPCR. Therefore the results are summarized in appendix 01 and 02 and not include in the results of the report. Furthermore the efficiency and the r^2 are in all cases not good enough and far above the maximum acceptable limits. Through the disappointing results of all the primer set combinations, the quantity of *TRHR* in the adrenal gland could not be evaluated.

Immunohistochemistry for detecting the *TRHR* in the pituitary gland and the adrenal gland

Several protocols are tested to find the most suitable for detecting the *TRHR* in the adrenal gland. The pituitary gland sections are used to evaluate the optimal protocol to expose the target epitope for the used antibody. These sections are used because of the known presence of the TRHR in the pituitary gland (15, 16).
First the citrate and TE medium are used at a temperature of 98°C for ten minutes and just only the TBS 0,025% Triton for ten minutes at room temperature. For the whole protocol see appendix 03. After staining, the TE coupe is stained too dark, the coupe with only TBS 0,025% Triton buffer is very light stained and the citrate coupe is unfortunately lost during the staining process. Through this result several other staining are performed to find the most suitable.
The second staining is also done with the TBS 0,025% Triton buffer. In this staining the TE and the citrate buffer are used as medium. Both pituitary and adrenal gland sections are used during this staining. (table 12)

Table 12: Medium, antibody dilution and used sections of the second staining

Medium	TE	TE	TE	TE	Citrate
Antibody dilution	1:75	1:100	1:150	Without antibody	1:100
Pituitary gland	X	X	X	X	X
Adrenal gland		X		X	

Pituitary gland staining 2
The section with TE 1:75 stains too dark and TE 1:150 stains too light. TE 1:100 is better compared with TE 1:75, however the background stained too much. The section without the TRHR antibody is clean, no cells are stained, as expected. The Citrate 1:100 section stains very dark and not specific.

Adrenal gland staining 2
In the TE 1:100 section, the cells of the medulla stain but the background too. The section without the TRHR antibody is clean, no cells are stained, as expected.

To reduce the background staining, the third staining is performed with a new buffer. The new buffer is TBS with 0,025% Triton and 0,1% Tween.

TE and citrate are again used as medium. Both the pituitary gland and the adrenal gland sections are used. Unfortunately this third staining did not work, all the sections are not stained. It is unclear why the sections did not stain. By this fact the staining is repeated with the same procedure. The fourth staining is therefore the same as the third (table 13). The results of the fourth staining are showed below.

Table 13: Medium, antibody dilution and used sections of the third and fourth staining

Medium	TE	Citrate	Citrate
Antibody dilution	1:100	1:150	1:200
Pituitary gland	X	X	X
Adrenal gland	X		

Pituitary gland staining 4
The section with TE 1:100 is very vague. Both the citrate 1:150 and 1:200 are too light.

Adrenal gland staining 4
In the TE 1:100 section the medulla is stained but vague and not specific.

In this fourth staining all the sections stained too light. So a fifth staining is performed with the same buffer but with the antibody less diluted (table 14).

Table 14: Medium, antibody dilution and used sections of the fifth staining

Medium	TE	TE	TE
Antibody dilution	1:50	1:75	1:100
Pituitary gland	X	X	X
Adrenal gland	X	X	X

Pituitary gland staining 5
In the section with TE 1:50 the anterior lobe is stained too dark, the middle lobe is not stained. The TE 1:75 is lighter stained and the TE 1:100 stained too light.

Adrenal gland staining 5
The TE 1:50 section stained overall brown but has an equal darker brown stained medulla, compared with an equal lighter stained cortex. The prominent visible connective tissue strands in the gland did not stain. Some groups of cells are stained darker compared with the rest of the gland. In the TE 1:75 section the cells are light stained and no background staining is visible anymore. The medulla stained much more lighter compared with the TE 1:50. The earlier mentioned connective tissue strands and

the darker stained group of cells visible in the TE 1:50 section are not visible in this section. The TE 1:100 section is too light.

To ensure the adrenal gland sections are stained properly and are not too light, the TE medium with an antibody dilution of 1:50 is used to evaluate the presence of the TRHR in different adrenal glands. The TBS 0,025% Triton 0,1% Tween buffer is used. The pictures of the stained sections are displayed in appendix 04.

Section 22A consist of two adrenal gland parts. In the first part several cells of the glomerulosa stained very dark. The rest of this part is equal slightly stained. The second part of the section is as a whole darker stained, compared with the first part. The glomerulosa is obvious darker stained compared to the rest of the cortex.

Section 22B stained overall light brown. However, in the section several blue regions of unstained groups of cells, are visible too.

In section P9280, the glomerulosa is clearly stained compared with the less stained fasiculata. Also the medulla is evenly stained. Nevertheless also several groups of cells in the fasiculata are darker stained compared with the rest of the fasiculata. (Figure 16).

In section 15/4 1 no medulla is visible. The section consist only of the adrenal cortex. Several groups of clearly dark stained cells are visible in the glomerulosa. The rest of the cortex is much lighter stained.

In section 15/4 2 the glomerulosa is slightly stained. The medulla is evenly stained, but darker compared with the glomerulosa. In the rest of the cortex several groups of stained cells are visible between the groups of unstained cells, which are blue (figure 17).

Section 7-4-14 consist of three slides of the same adrenal gland. Therefore two slides are stained with the TRHR antibody and one is stained without putting the TRHR antibody on the sections during the procedure. In the slides with the TRHR antibody the medulla and glomerulosa stain brown, also several groups cells of the rest of the cortex stain brown. The section without the TRHR antibody is not stained, no brown cells are visible. All the cells are blue as expected (figure 18 and 19).

The glomerulosa of section 11B is stained dark brown. Also several dark brown dots are visible next to the nucleus of the cells of the rest of the adrenal cortex. These dots give the section a stippled appearance when the section is examined without the microscope.

Section 2276 was very small at the beginning of the procedure. During the staining procedure it got stuck and the tissue was too much damaged to evaluate. Therefore no information is available about this section.

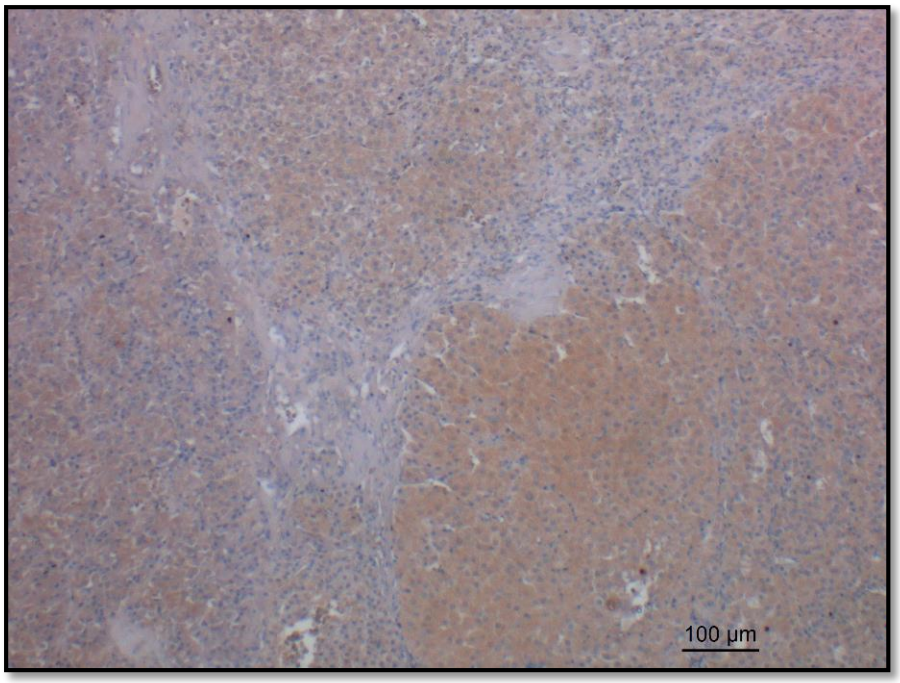

Figure 17: 15/4 2 In the cortex several groups of stained cells are visible between the groups of unstained cells, which are blue

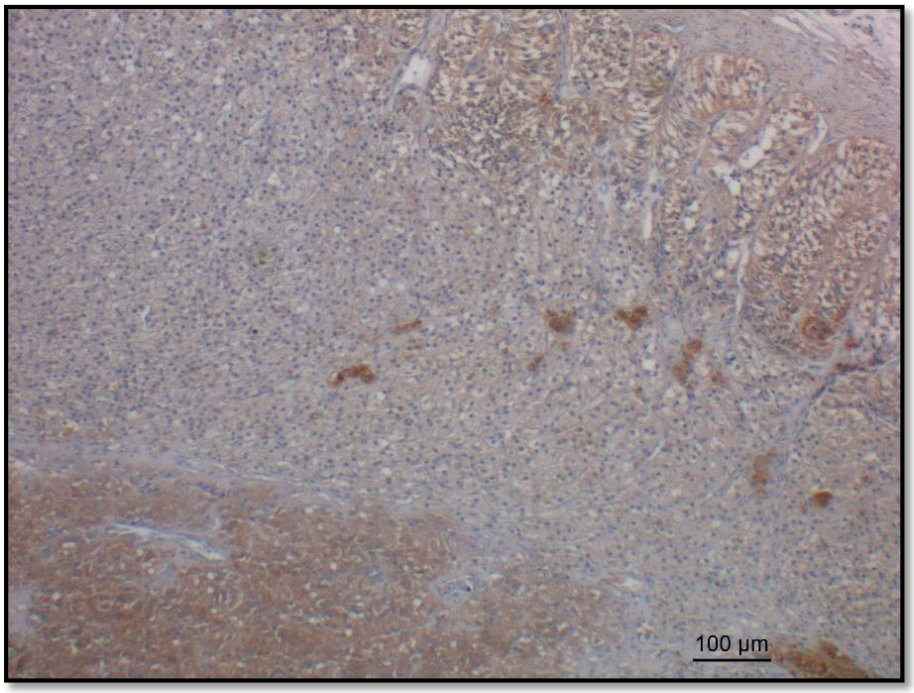

Figure 18: 7-4-14 With the TRHR antibody, medulla and glomerulosa stain brown, also several groups of cells of the rest of the cortex stain brown

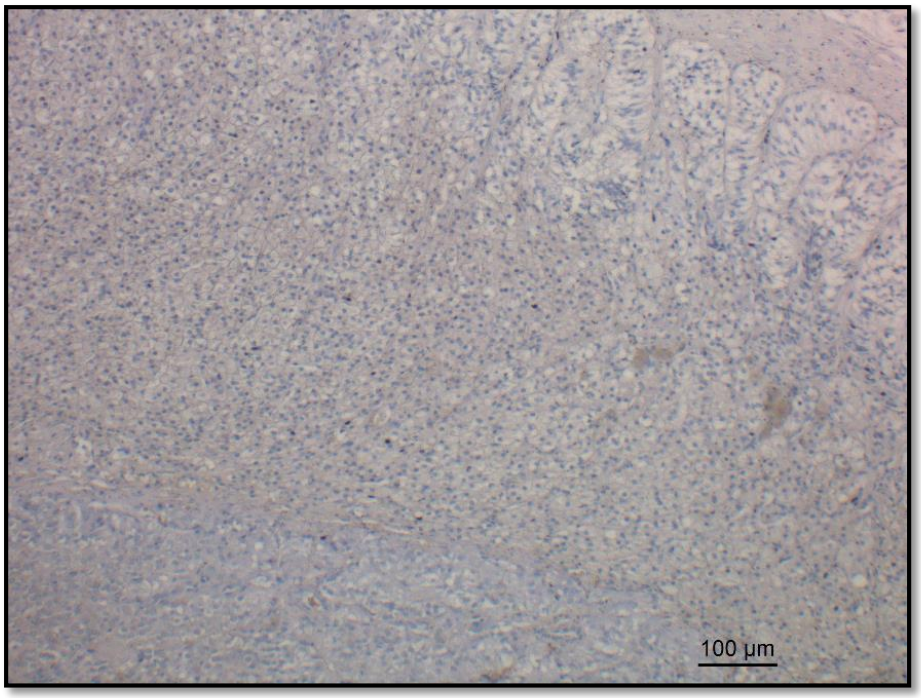

Figure 19: 7-4-14 Without the TRHR antibody, the cells are unstained and remain blue, no brown cells are visible

Comparison of the pituitary gland sections and the adrenal gland sections

The pituitary gland sections and adrenal gland sections are also compared with each other. The sections which are compared underwent the same protocol and the sections are stained with an antibody dilution of 1:50 (figure 20). The pituitary gland section (left) is much darker compared with the adrenal gland section (right).

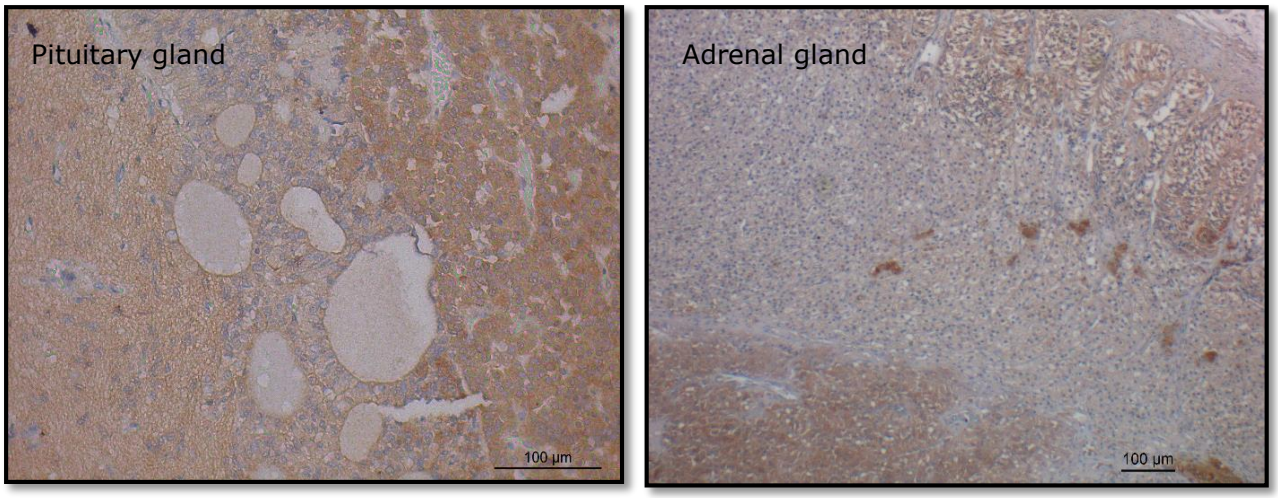

Figure 20 Pituitary gland (left) and adrenal gland (right) stained with *TRHR* antibody dilution 1:50.

Discussion

With this research the expression of the *TRHR* in the canine adrenal gland is proven in different ways. After PCR, electrophoresis and sequencing at least in two of the six adrenal gland samples the expression of the *TRHR* is proven. Only these two samples are sequenced because they gave the brightest bands after electrophoresis. The bands of the other samples aren't bright enough to sequence. The two sequenced samples are from the only two female dogs, the remaining four samples are from male dogs. Further research to evaluate the difference between male and female is a logical and nice addition to this research, to evaluate the result of this research is a coincidence or there is really a difference between female and male dogs.

In the first experiment six different adrenal glands are used for RNA isolation. Only 30 milligrams is used in the procedure. Therefore it is possible only the cortex is used instead of the cortex and medulla. Thereby PCR and sequencing prove the presence of the *TRHR* in the cortex but provides no information about the *TRHR* in the medulla. Further research to evaluate the difference between the cortex and medulla would be a nice addition to this part of the research.

With the first PCR and sequencing the presence of only a small fragment of 27 bases of the *TRHR* region in the adrenal gland is confirmed. The alignment with the canine genome of this fragment is 100,00%. With new primers a longer fragment of 636 bases is produced to evaluate a bigger part of the *TRHR*. From this product a part of 257 bases is successfully sequenced, with an alignment of 91,05% with the canine genome. The 9% mismatches are due to sequencing errors. Due to background and low concentration of the product, it was not possible to sequence the whole expected product of 636 bases. With these results the presence of the *TRHR* in the adrenal gland is proven. Still only a small part of the *TRHR* is sequenced and not the whole receptor. Further research could involve sequencing and comparing with the canine genome of the whole cDNA of the receptor.

PCR also indicates the expression of *TRHR* is less in the adrenal gland compared with the pituitary gland. Furthermore in not every tested adrenal gland sample a clear visible band after electrophoresis is present. The lack of a clear band can be caused by the small amount of *TRHR* in canine adrenal glands. After replication with PCR the amount is still too small to express a bright band. A possibility for showing no band is a variation in the population, which give some dogs a *TRHR* in the adrenal gland and some not. The variation can only explain the absence of the bands but cannot explain the vague and light bands. Due to the lack of clear bands in several samples, only two of the samples are used for sequencing and only in these samples the appearance of the *TRHR* in the adrenal gland is proven. Several of the bands which are visible after electrophoresis are all at the same height. Therefore these samples give no indication for isoforms like in other species.
In the research by P. de la Pena, et al (1992) in rat anterior pituitary cells two different isoforms of the thyrotropin-releasing hormone receptor are found. The two isoforms are generated by an alternative splicing. The second splice variant has a deletion of 52-bases which result in a smaller fragment and a different amino acid sequence (13, 17). Also in pigs different isoforms are known. In the research by X. Jiang et al, (2011) three splice variants of the porcine *TRHR* are isolated. Also in this research the presence of the

TRHR in all kind of tissues has been examined. This research proofed the presence of *TRHR* transcript in several porcine tissues, including pituitary, hypothalamus, fat tissue and testis. The *TRHR* is not observed in the thyroid, liver and spleen. The adrenal gland was not included in this research (18). When looked at the canine *TRHR*, as earlier mentioned, only one splice variant is known. The canine *TRHR* gene is located at chromosome 13, has one transcript, 73 orthologues and eight paralogs. It consist of two exons with in between a large intron. The exons together have a length of 1582 base pairs witch give rise to a product of 398 amino acids (13). The is a seven transmembrane spanning receptor and belongs to the G protein-coupled receptor superfamily. (18, 19)

To measure the exact amount of the *TRHR* in the adrenal gland qPCR is necessary. First several reference genes are used and also several gradients of 55-65°C are performed. Unfortunately the results of the gradients were disappointing and no qPCR could be performed. Hereby no information about the exact amount of *TRHR* is available. More research to evaluate the exact amount of the *TRHR* would be recommended.

Previous research with rats suggests the presence of *TRHR* mRNA in the anterior lobe of the pituitary gland. The *TRHR* mRNA is also in a lot of other tissues found, such as heart, spleen, liver and lung. In the stomach, small intestinal, colon, adrenal gland, testis and pancreas the receptor is found by immunohistochemistry (15).
In another research, done by S. Konaka et al (1997), 61,1% of the cells of the anterior lobe of the pituitary gland expressed the *TRHR* mRNA transcript (16). With these results the expectation is the same about the presence of the TRHR in the canine pituitary gland. Therefore the canine pituitary gland is used to evaluate the immunohistochemistry protocol.

Section 7-4-14 consists of three slices of the same adrenal gland, one is stained without TRHR antibody and two are stained with antibody. Unlike the section without TRHR antibody (which is blue), in the sections with antibody the medulla, glomerulosa and several groups of cells in the rest of the cortex stain brown. This contrast indicates the antibody does not attach to every cell, but to specific (parts of) cells. According to the manufacturer, this specific part is the TRHR. Therefore this contrast in staining is due to the presence of the TRHR and not due to background staining (figures 18 and 19). The staining of the sections indicates the attachment of the rabbit anti-antibody to the TRHR antibody which is attached to the TRHR in the adrenal gland. Hereby the immunohistochemistry results indicate the presence of the TRHR in the adrenal gland. The TRHR was visible in the cortex and the medulla. The medulla stained sometimes even darker compared with the cortex, which indicates the medulla contains in some cases more TRHR compared with the cortex. The more brown staining is visible the more antibodies have attached. Not every cell of the adrenal gland sections possess the receptor. This is clearly visible in section 15/4 2 (figure 17). In this section in the cortex several groups of stained cells are visible between the groups of unstained cells, which are blue. These blue cells do not possess the TRHR and thereby do not stain brown.

Based on the darkening of the sections, also with immunohistochemistry the amount of TRHR in the adrenal gland is less compared with the pituitary gland. The pituitary gland stained much darker with the same antibody dilution of 1:50 compared with the adrenal gland (figure 20).

A big difference in staining of the different zones of the cortex is also very clear. The glomerulosa is in many cases the most stained compared with the fasiculata and reticularis. Despite this big difference also in several sections multiple groups of dark stained cells are also visible in the fasiculata. Furthermore the fasiculata and reticularis are light stained. These findings are in contrast with the expectation. The zona fasiculata and reticularis are responsible for the secretion of cortisol. With this fact the TRHR is more expected in these zones instead of the glomerulosa. Further research is necessary to further evaluate this fact.

Conclusion

With this research the expression of *TRHR* in the canine adrenal gland is proven in different ways. After performing PCR and sequencing, the alignment of the *TRHR* in the adrenal gland with the canine genome is between 91.05%-100,00%. The 9% mismatches are due to sequencing errors. The PCR indicates the concentration of *TRHR* mRNA in the adrenal gland is less compared with the pituitary gland. The qPCR to quantify the amount of *TRHR* failed due to the lack of quality and purity of the products. No information about the quantity of the *TRHR* can be given. The clinical relevance of the amount of *TRHR* in the adrenal gland is also unclear and had to be further examined. Furthermore, immunohistochemistry indicate the presence of the TRHR in the adrenal glands. Based on the results of the immunohistochemistry the TRHR is present in different parts of the adrenal gland. TRHR is more expressed in the glomerulosa and medulla compared with the fasiculata and reticularis. Also immunohistochemistry indicates the presence of TRHR in the adrenal gland is less compared with the pituitary gland. After this research more is clear about the TRHR expression in the adrenal gland. However further research is necessary to provide more information.

Further research

Due to a lack of purity and quality, the qPCR could not be performed. No information about the quantity of the *TRHR* in the adrenal gland can be given. In further research the quantity can be measured and compared with other tissues such as the pituitary gland. When the quantity is known the clinical relevance can also be examined.

After PCR two of the six adrenal gland samples gave clear bands at the expected height of the *TRHR*. These two samples were the only two of female adrenal glands. The remaining were male adrenal glands. Further research to determine the difference between female and male dogs would be a nice addition to this research.

During PCR probable only cortex is used. Thereby only the presence of the *TRHR* in the cortex is proven and no information about the medulla is available. After immunohistochemistry a clear difference between medulla and cortex is visible. The medulla is often more stained compared with the cortex. In the cortex also a clear difference between the different zones is visible after immunohistochemistry. Further research to the difference between cortex and medulla with PCR and with immunohistochemistry and between the different zones with immunohistochemistry would be a nice addition to this research.

In this research only a small fragment of the *TRHR* is sequenced. In further research the whole receptor could be sequenced to see if it is similar in different dogs. Since in not every sample, used in the electrophoresis, a clear band representing the *TRHR* fragment was visible, further research could give more information about the variations between the dogs and exclude the possibility of an incidental finding of the light and almost not visible *TRHR* fragments in some of the used adrenal glands.

Also the effect of binding of TRH to the TRHR can be evaluated. Further research to the mechanism in which binding of TRH can influence the cortisol stimulation and secretion would be a nice addition to this research.

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Pictures used in the introduction

H.S. Kooistra: H.S. Kooistra. Personal communication June-November 2015

VCA Animal Hospitals and BPCC: http://www.vcahospitals.com/main/pet-health-information/article/animal-health/adrenal-medulla-tumors/233 and https://my.bpcc.edu/content/blgy225/Hormones/Hormones_print.html

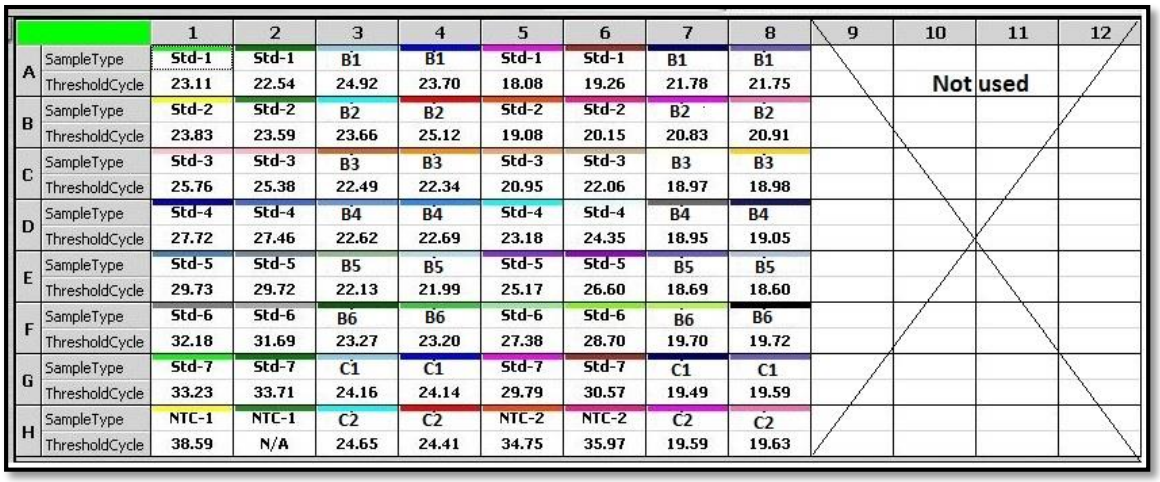
Appendix 01 Outcome qPCR reference genes

Table 01 qPCR threshold of the reference genes RPL13, RPS19, SDHA*

		1	2	3	4	5	6	7	8	9	10	11	12
A	SampleType	Std-1	Std-1	B1	B1	Std-1	Std-1	B1	B1	Std-1	Std-1	B1	B1
	ThresholdCycle	18.20	17.93	19.61	19.76	17.62	17.72	18.67	18.64	22.68	22.82	24.77	25.53
B	SampleType	Std-2	Std-2	B2	B2	Std-2	Std-2	B2	B2	Std-2	Std-2	B2	B2
	ThresholdCycle	19.18	19.16	19.02	19.16	18.82	18.98	18.71	18.78	23.59	23.71	24.04	24.24
C	SampleType	Std-3	Std-3	B3	B3	Std-3	Std-3	B3	B3	Std-3	Std-3	B3	B3
	ThresholdCycle	21.12	21.09	17.80	17.79	20.82	21.09	17.75	17.82	25.78	25.76	22.87	22.89
D	SampleType	Std-4	Std-4	B4	B4	Std-4	Std-4	B4	B4	Std-4	Std-4	B4	B4
	ThresholdCycle	23.38	23.38	17.44	17.49	22.89	23.01	17.56	17.71	27.96	27.72	22.31	22.35
E	SampleType	Std-5	Std-5	B5	B5	Std-5	Std-5	B5	B5	Std-5	Std-5	B5	B5
	ThresholdCycle	25.40	25.51	17.64	17.50	25.04	25.07	17.78	17.70	29.97	29.96	22.57	22.74
F	SampleType	Std-6	Std-6	B6	B6	Std-6	Std-6	B6	B6	Std-6	Std-6	B6	B6
	ThresholdCycle	27.22	27.38	17.68	17.84	26.89	27.24	17.83	17.84	32.62	32.63	23.33	23.54
G	SampleType	Std-7	Std-7	C1	C1	Std-7	Std-7	C1	C1	Std-7	Std-7	C1	C1
	ThresholdCycle	29.60	29.51	19.51	19.62	29.25	29.08	19.77	19.80	33.24	34.54	23.01	23.36
H	SampleType	NTC-1	NTC-1	C2	C2	NTC-2	NTC-2	C2	C2	NTC-3	NTC-3	C2	C2
	ThresholdCycle	N/A	36.05	21.04	20.94	N/A	N/A	20.45	20.45	N/A	N/A	23.99	25.74

*RPL13: lane 1-4, RPS19: lane 5-8, SDHA: 9-12.

Table 02 qPCR threshold of the reference genes HPRT and GAPDH*



		1	2	3	4	5	6	7	8	9	10	11	12
A	SampleType	Std-1	Std-1	B1	B1	Std-1	Std-1	B1	B1				
	ThresholdCycle	23.11	22.54	24.92	23.70	18.08	19.26	21.78	21.75		Not used		
B	SampleType	Std-2	Std-2	B2	B2	Std-2	Std-2	B2	B2				
	ThresholdCycle	23.83	23.59	23.66	25.12	19.08	20.15	20.83	20.91				
C	SampleType	Std-3	Std-3	B3	B3	Std-3	Std-3	B3	B3				
	ThresholdCycle	25.76	25.38	22.49	22.34	20.95	22.06	18.97	18.98				
D	SampleType	Std-4	Std-4	B4	B4	Std-4	Std-4	B4	B4				
	ThresholdCycle	27.72	27.46	22.62	22.69	23.18	24.35	18.95	19.05				
E	SampleType	Std-5	Std-5	B5	B5	Std-5	Std-5	B5	B5				
	ThresholdCycle	29.73	29.72	22.13	21.99	25.17	26.60	18.69	18.60				
F	SampleType	Std-6	Std-6	B6	B6	Std-6	Std-6	B6	B6				
	ThresholdCycle	32.18	31.69	23.27	23.20	27.38	28.70	19.70	19.72				
G	SampleType	Std-7	Std-7	C1	C1	Std-7	Std-7	C1	C1				
	ThresholdCycle	33.23	33.71	24.16	24.14	29.79	30.57	19.49	19.59				
H	SampleType	NTC-1	NTC-1	C2	C2	NTC-2	NTC-2	C2	C2				
	ThresholdCycle	38.59	N/A	24.65	24.41	34.75	35.97	19.59	19.63				

* HPRT: lane 1-4, GAPDH: lane 5-8

**Due to a mistake of putting twice the amount of the standard line in the first standard line (lane 5) these thresholds are 1,00 point lower than there duplo (lane 6). Therefore only lane 6 is used to define the standard line of reference gene GAPDH.

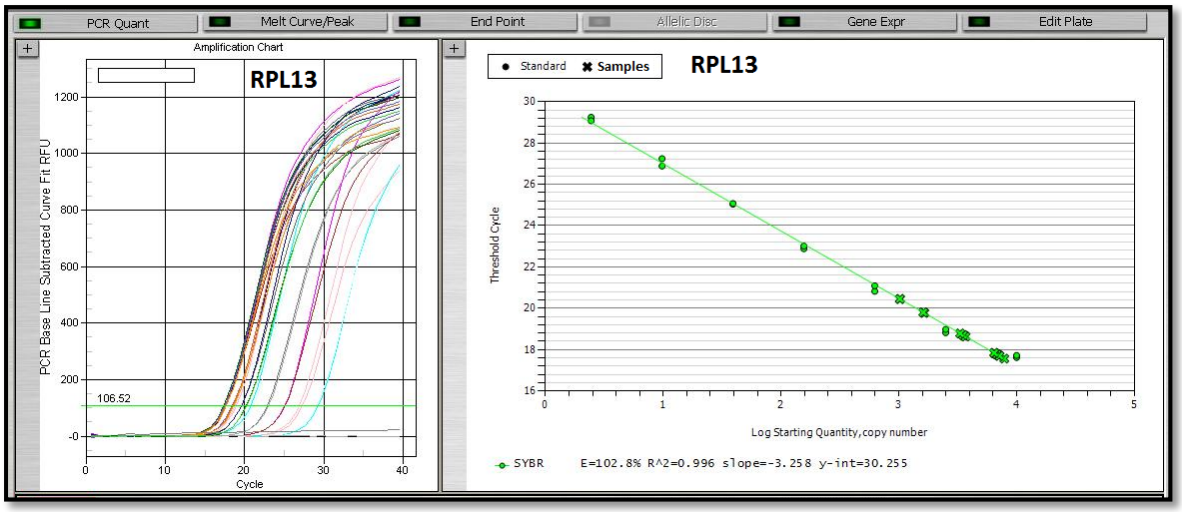


Figure 01 Amplification chart, E-value and r^2 of reference gene RPL13

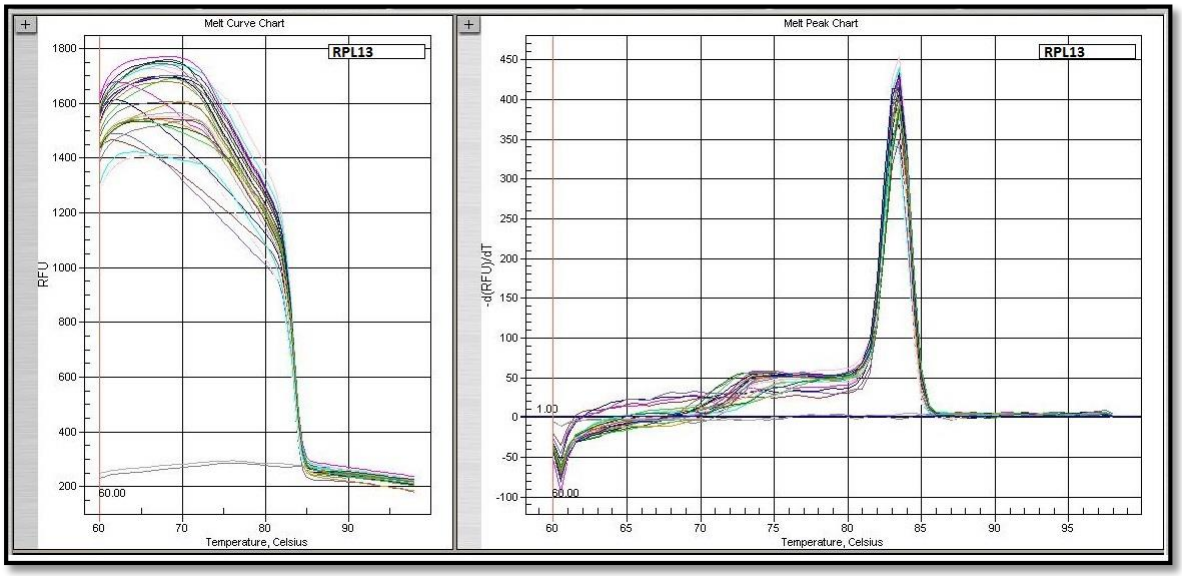


Figure 02 Melt curve and melt peak of reference gene RPL13

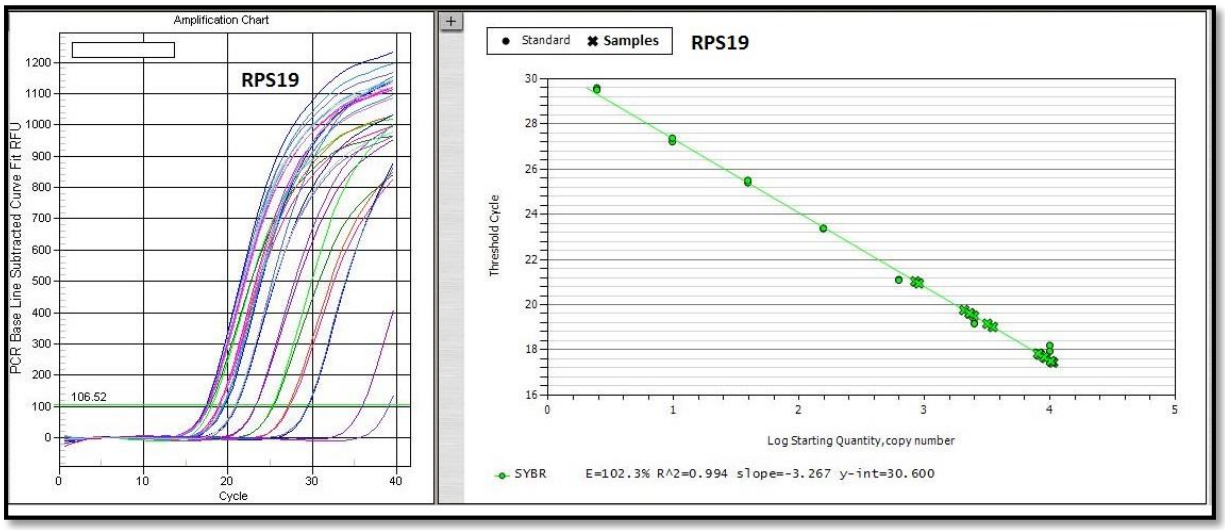


Figure 03 Amplification chart, E-value and r^2 of reference gene RPS19

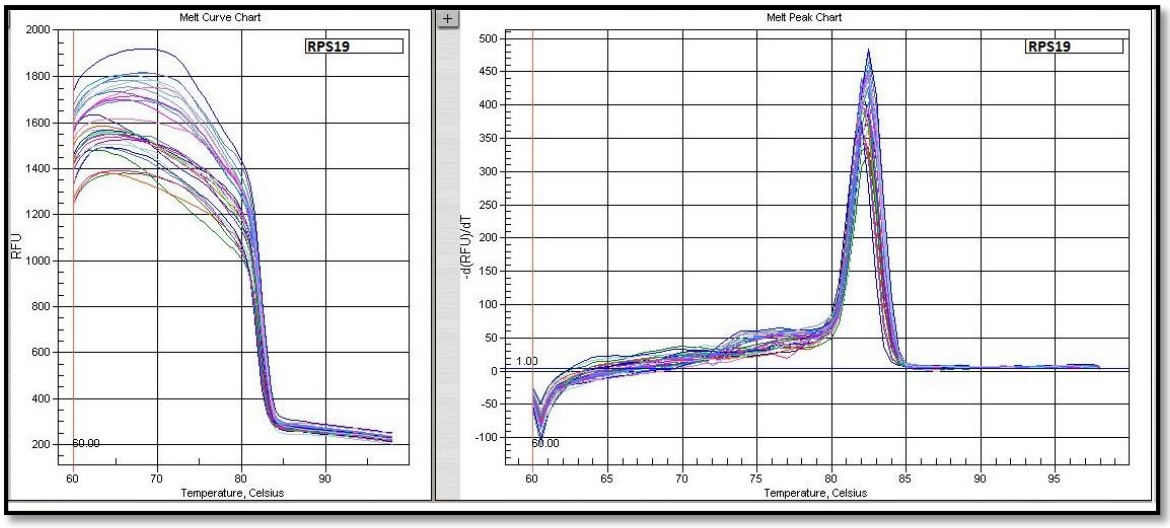


Figure 04 Melt curve and melt peak of reference gene RPS19

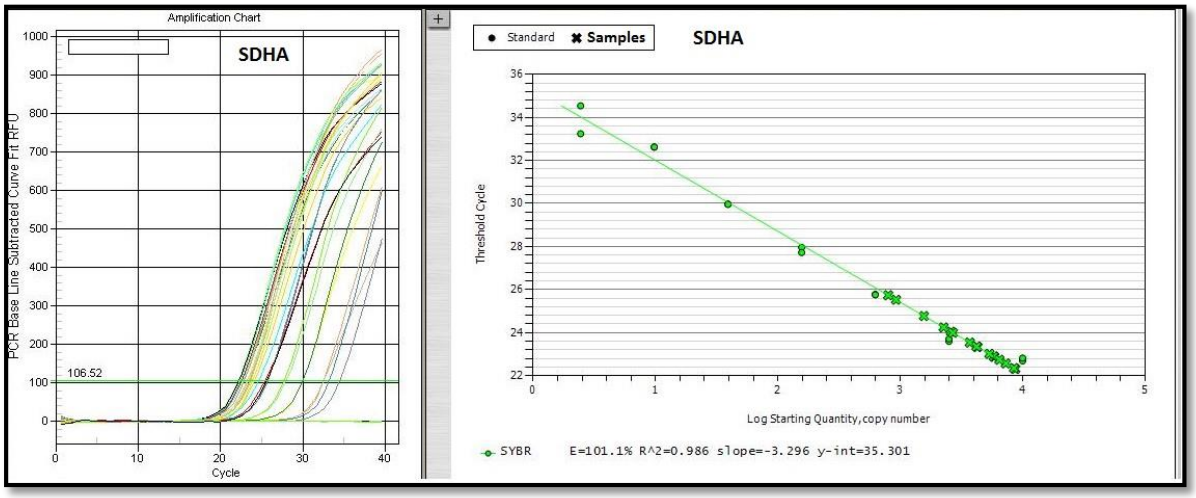


Figure 05 Amplification chart, E-value and r^2 of reference gene SDHA

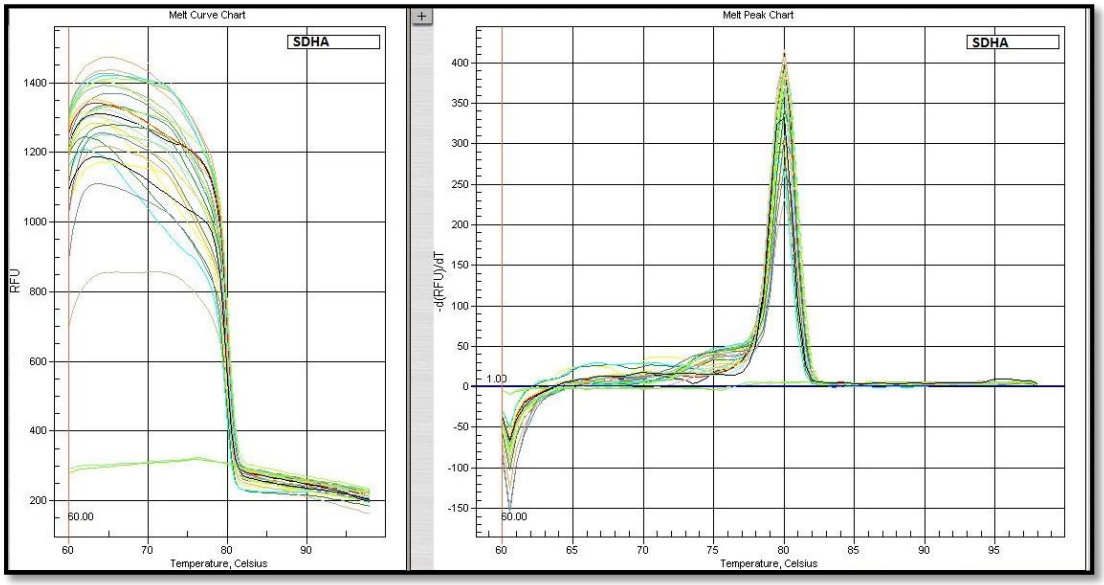


Figure 06 Melt curve and melt peak of reference gene RPS19

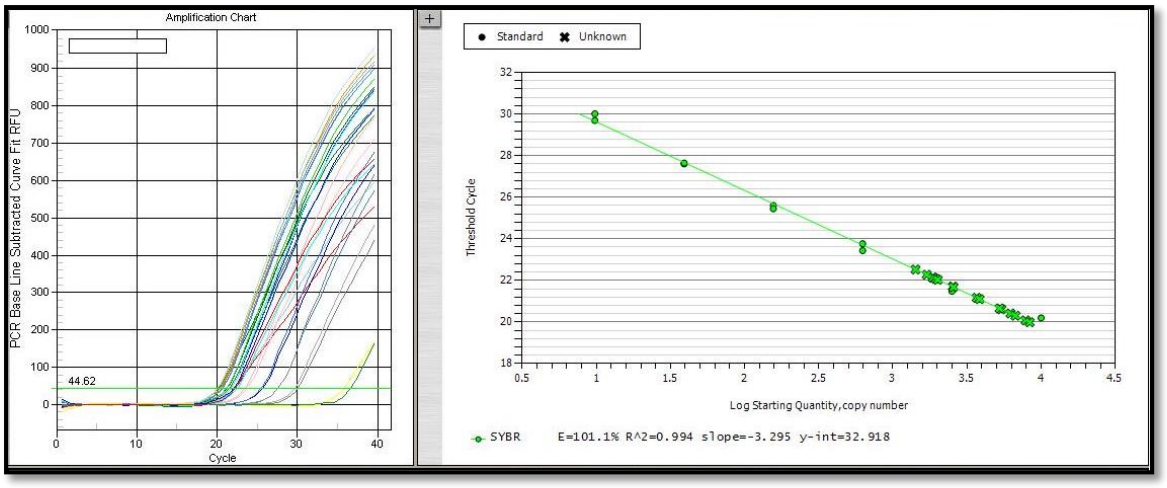


Figure 07 Amplification chart, E-value and r^2 of reference gene HPRT

**To establish the E-value of 101,1% sample S1, S2 and S7 of the first standard line and S7 of the second standard line were not include in the analysis.*

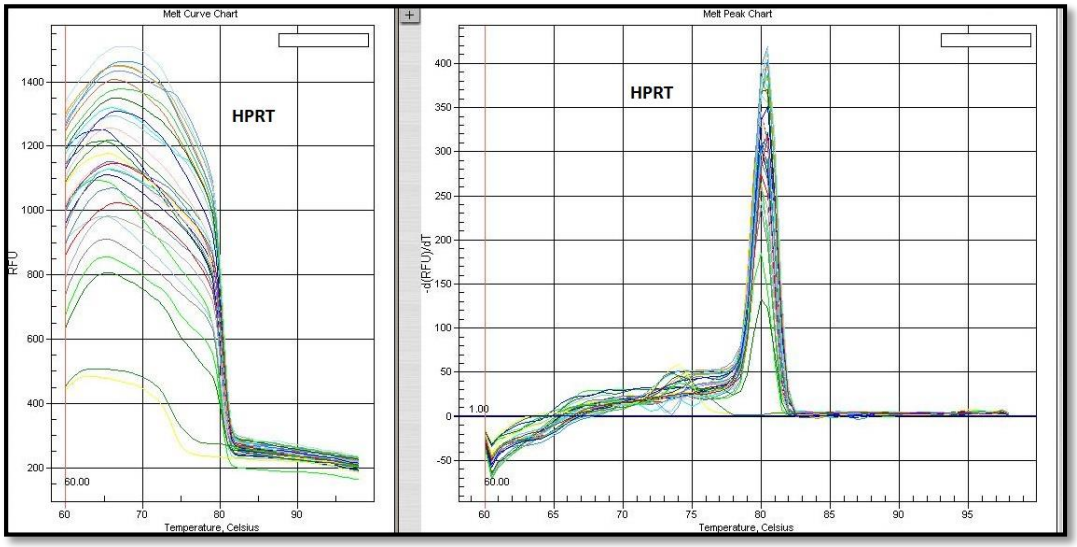


Figure 08 Melt curve and melt peak of reference gene HPRT

**The peak before the melt peaks is caused by the dimers in the NTC.*

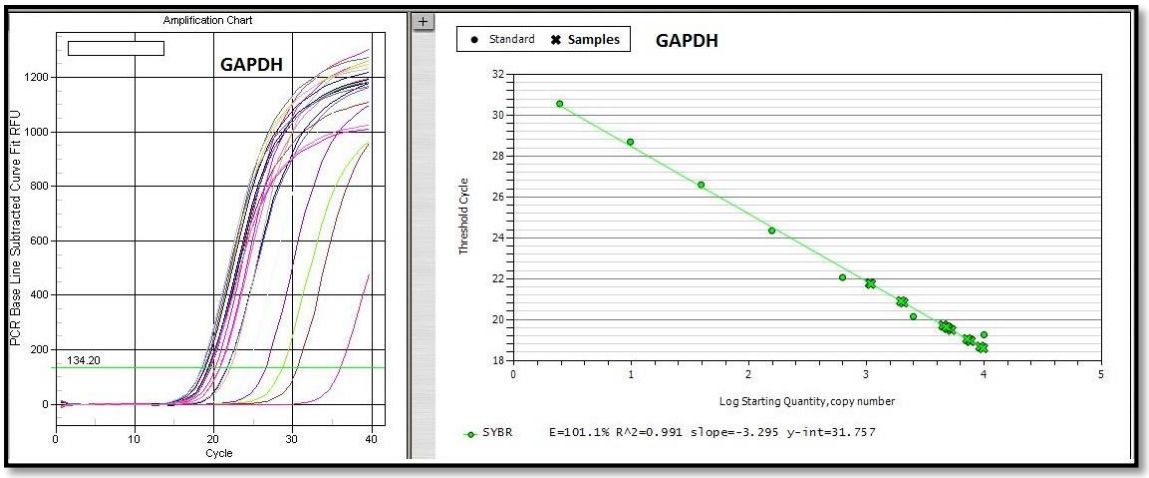


Figure 09 Amplification chart, E-value and r^2 of reference gene GAPDH

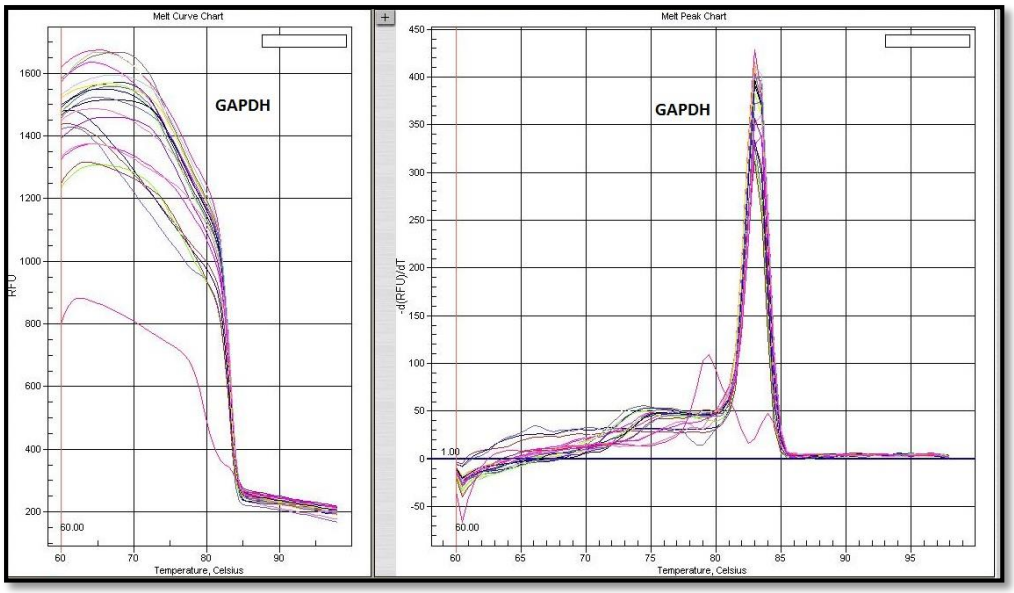


Figure 10 Melt curve and melt peak of reference gene GAPDH

**Only the second standard line is used due to a pipette mistake in the first standard line.*

***The peak before the melt peaks is caused by the dimers in the NTC.*

Appendix 02 Melt curves of the used primer sets

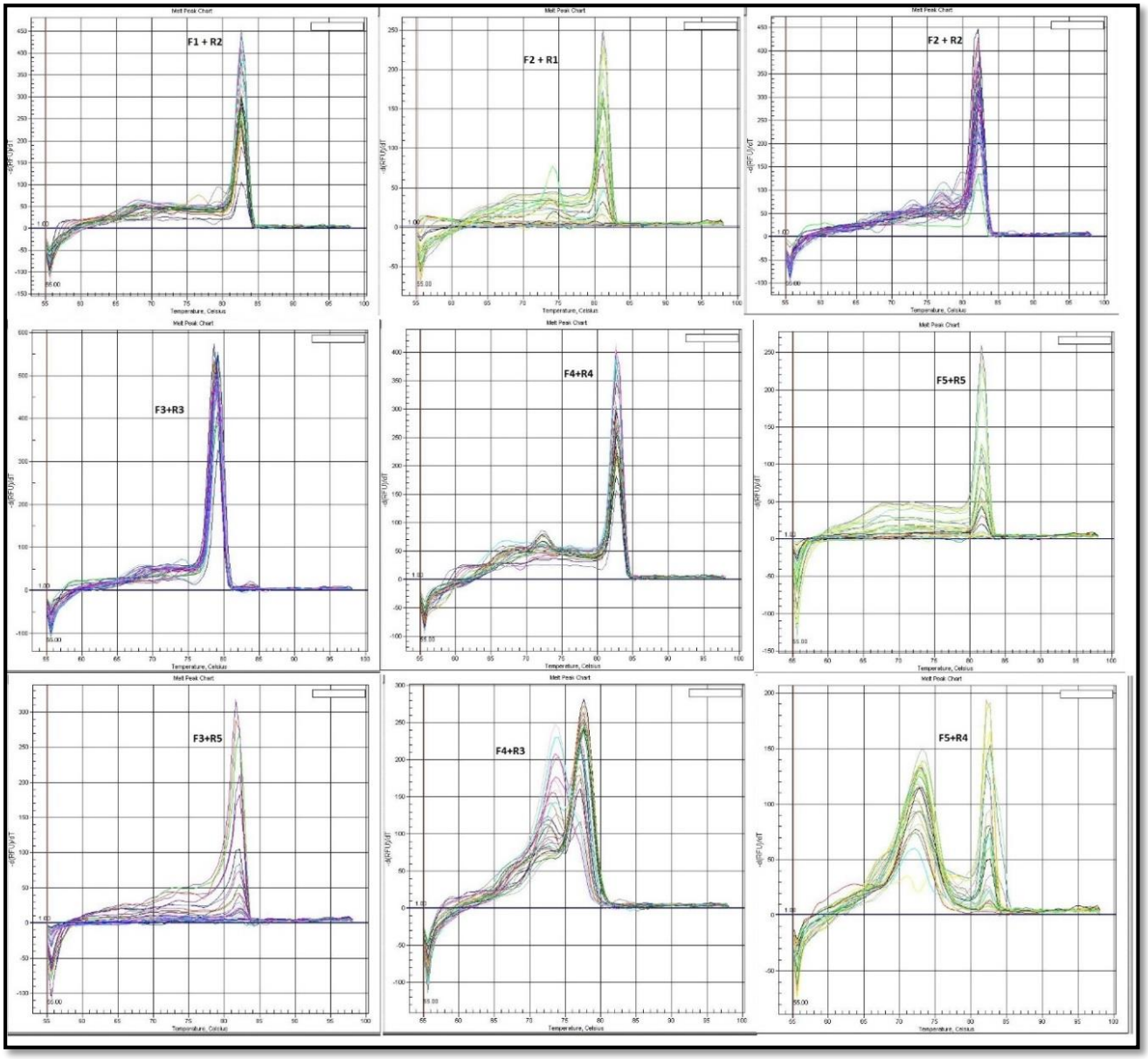


Figure 11 Melt peaks of the gradients with the different primer combinations.

Appendix 03 Protocol for immunohistochemistry

ʻ = minute
ʻʻ = second

2x 5ʼ in xylene
1x 3ʼ 96% alcohol
1x 3ʼ 80% alcohol
1x 2ʼ 70% alcohol
1x 2ʼ 60% alcohol

2x 5ʼ buffer TBS 0,025% Triton 0,1% Tween (= The buffer meant in the rest of the protocol)

Treatment for each section is different to see which one works the best

- 1 section: 10ʼ citrate (pH 6) 98°C
- 1 section: 10ʼ TE (pH 9) 98°C
- 1 section: 10ʼ in the buffer at room temperature

➔ Only TE is used to stain the adrenal gland sections

20ʼ cool off at room temperature
2x 5ʼ buffer
30ʼ 10%NGS in 1%BSA in buffer

Overnight with the first antibody (Anti-TRH Receptor antibody (ab72179))

- Overnight 1st AL 1:50 in the buffer with 1% BSA at 4°C

2x 5ʼ buffer
10ʼ Dual endogenous enzyme block S2003 (DAKO®)
2x 5ʼ buffer

30ʼ 2th Antibody (rabbit)
2x 5ʼ buffer

DAB 5-10 minutes (depends on the rate of coloring)
Washing with mQ (in the cub on the bench)

30ʼʼ Heam… (different staining)
10ʼ washing in the sink with running water

2ʼ 60% alcohol
2ʼ 70% alcohol
3ʼ 80% alcohol
2x 3ʼ 96% alcohol
5ʼ 96% alcohol
2x 5ʼ xylene

Include with mounting medium

Appendix 04 Immunohistochemistry of the adrenal gland sections

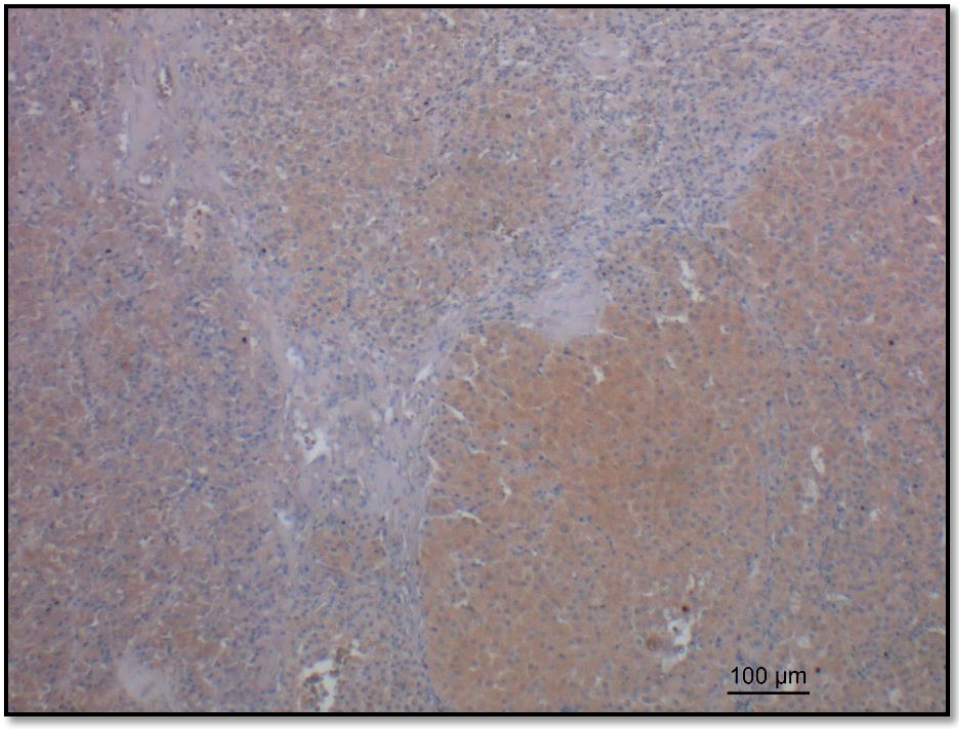


Figure 12: 15/4 2 In the cortex several groups of stained cells are visible between the groups of unstained cells, which are blue

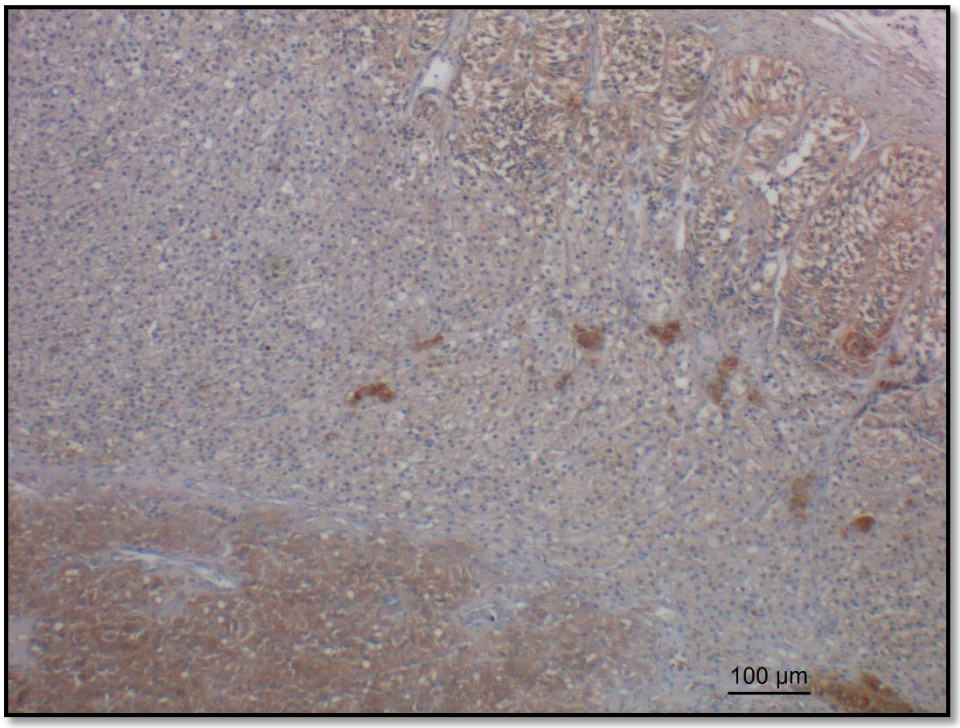


Figure 13, 7-4-14 With the TRHR antibody, medulla and glomerulosa stain brown, also several groups of cells of the rest of the cortex stain brown

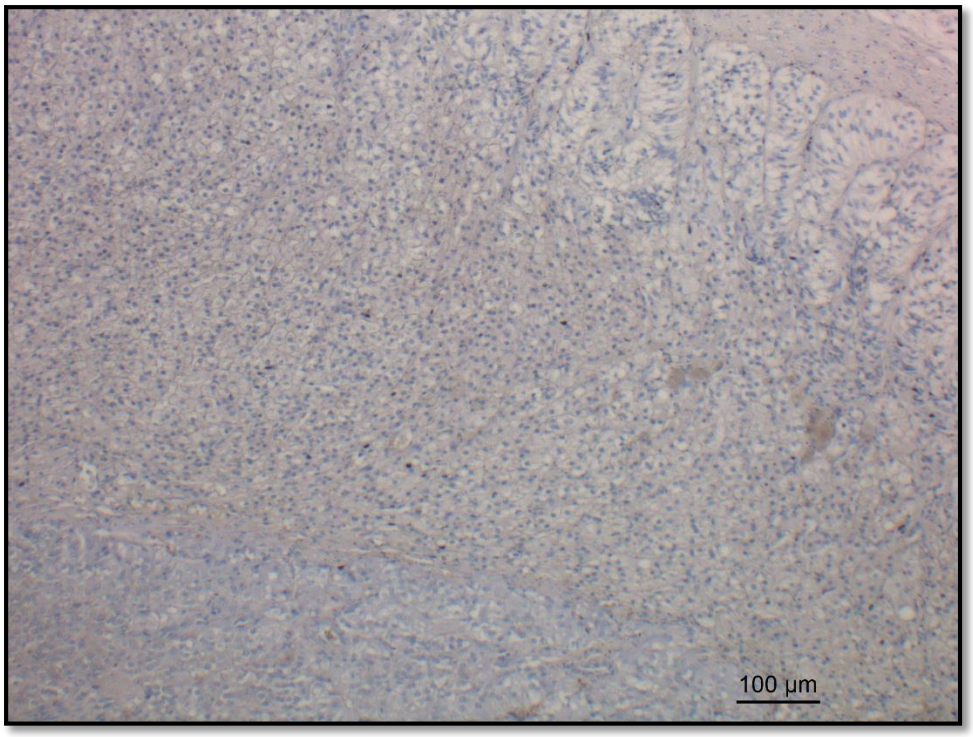


Figure 14, 7-4-14 Without the TRHR antibody, the cells are unstained and remain blue, no brown cells are visible

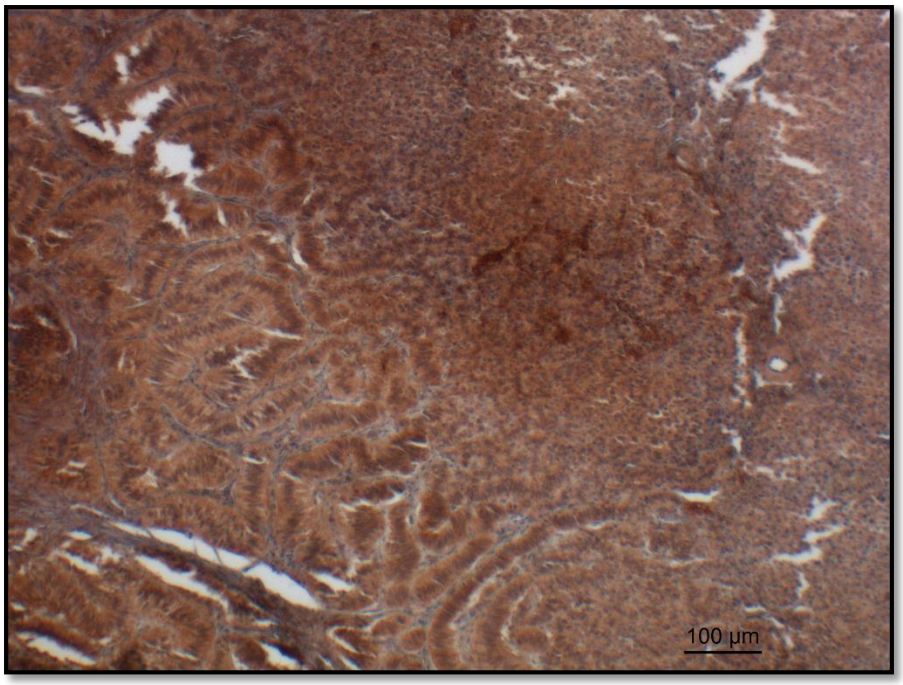


Figure 15, 11B The glomerulus is stained really dark

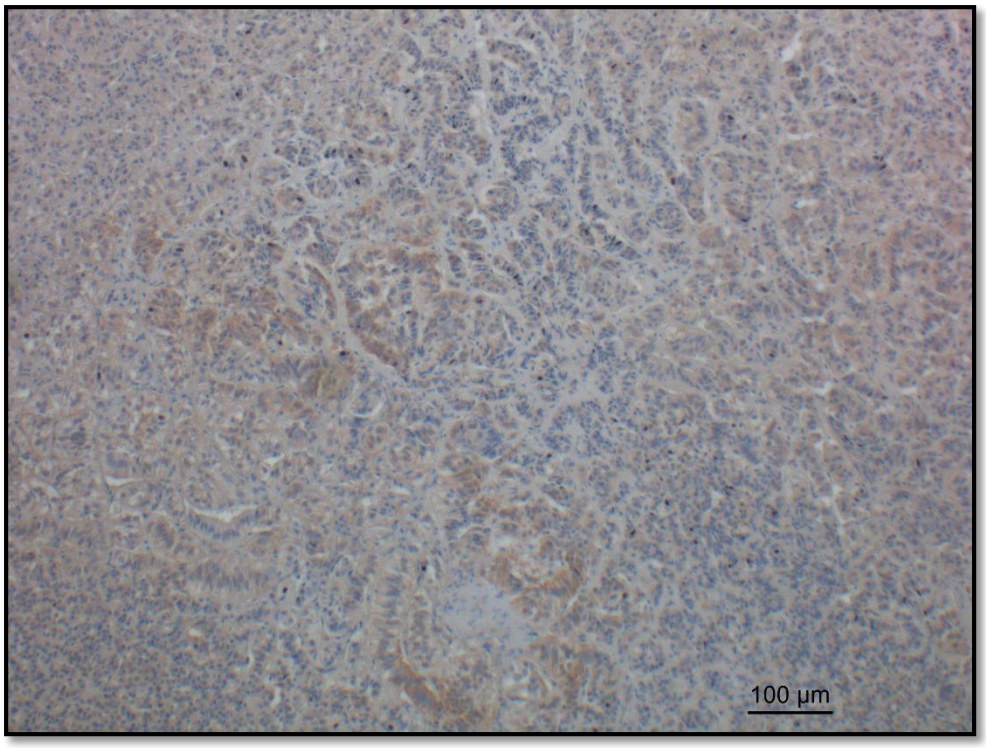


Figure 16, 15-4 1 Several group of cells of the cortex are stained. Also several cells are unstained (blue)

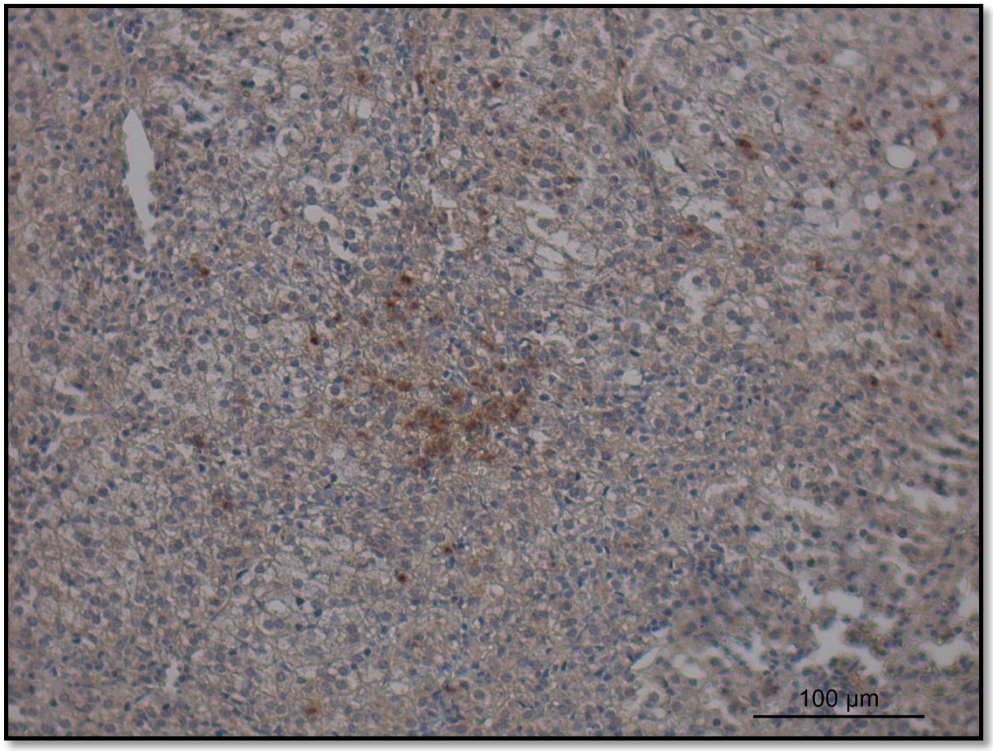


Figure 17, 22B Some blue group of cells are visible between the brown stained cells

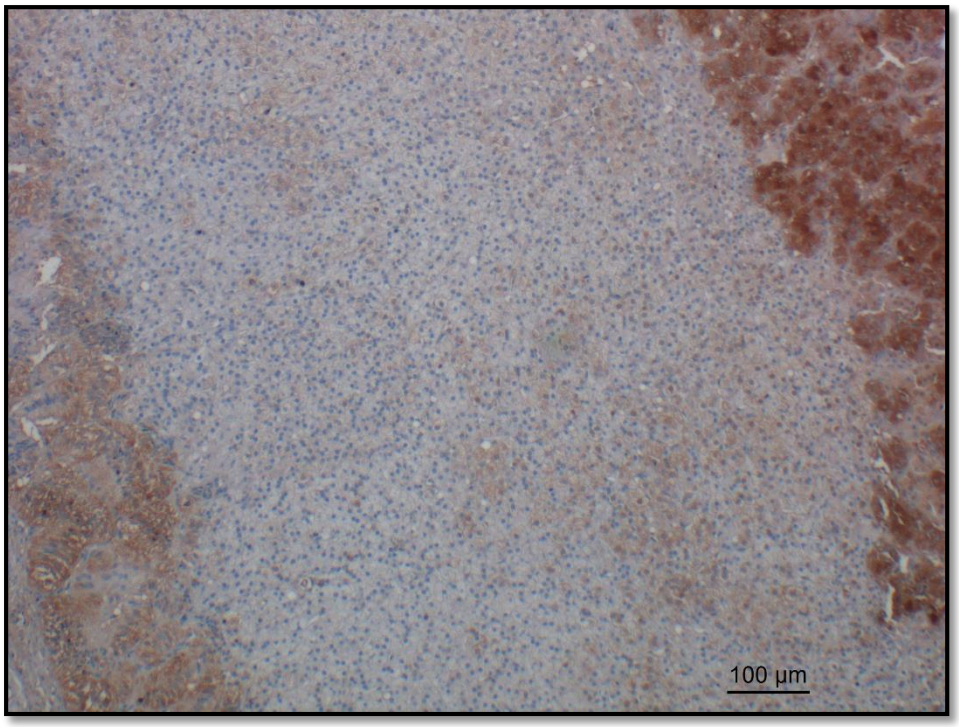


Figure 18, 9250 The glomerulosa and medulla are clearly stained compared with the less stained fasiculata and reticularis