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A study of candidate genes for anxiety in the Havanese breed

Et kandidatgenstudie på angst hos Bichon Havanais

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Preface

As a Havanese owner and breeder I appreciate the physical and mental characteristics of the breed. Zoila Portuondo Guerra is a Cuban Havanese breeder, founder and first President of the Havanese Club of Cuba and one of the founders of the Cuban Kennel Club. About Havanese temperament she writes:

"The temperament of the Bichon Havanese plays a decisive role in its form. These dogs should be neither timid nor aggressive. By character, the breed is lively, intelligent and up to any situation. It shows no cowardice, in spite of its size. (...) It gets along well with others of the same breed and even other breeds; for, although the Bichon Havanese is somewhat dominant, it's not a quarrelsome dog. "

The temperament is one of the most characteristic traits of the Havanese and one of the main reasons I acquired the breed in the first place. When I got my first Havanese in 2006, the breed was still quite rare in Norway. All the first Havanese that I acquainted, including my founder bitch, had the gentle, calm and dominant temperament that is described by Zoila Portuondo Guerra. However, in later years I have become increasingly aware of the anxiety issues some Havanese are displaying.

Through personal encounters with numerous Havanese in the show ring, and personal experience through breeding these dogs, I have seen large variation in the temperament of different Havanese and the level of anxiety expressed. Reviewing hundreds of dog show critiques also reveals that some Havanese have a timid personality that is not breed-typical.

Anxiety in Havanese

The quotes below are examples of posts found in a Norwegian Havanese group on Facebook between October 2013 and January 2015. The posts are translated and anonymised.

"Has anyone else had issues with their Havanese being terrified of all new people and dogs? ... I have tried to socialize her, but with no effect. She is still afraid. She has had NO bad experiences with anyone..."

"My dog has suddenly decided that all big dogs are scary. Although he has always been a bit skeptical, he used to be fine once he warmed up to them. Lately though, he has been very scared when big dogs show signs of wanting to play with him. He screams regardless of whether the other dog is even in direct contact with him. How can I handle this? He has had no experiences that could explain why he is this scared now."

"My dog is so stressed out lately. She growls and barks for no apparent reason..."

These quotes illustrate that there are indeed anxious individuals within the breed. They also show some of the stress and welfare issues these behavioral problems are causing to the animals and owners.

In 2012/2013, the rest of the Breeding Advisory Panel of The Norwegian Havanese Club (NBHK) and I, created a questionnaire about health and temperament in the Havanese. 16,8% of owners stated their dog was "a little nervous" or "very nervous". This confirmed the concerns I

have had with regards to the prevalence of anxiety in the Havanese – a breed that I initially purchased and started to breed because of their excellent temperaments. My personal opinion is that anxiety is one of the main concerns in Havanese and several other breeds alike. I believe genetic research on behavior in dogs is crucial to better their welfare. This is what motivated me to study anxiety in Havanese. The possibility to study genetics of behavior in the end of the studies at the Veterinary faculty was a great opportunity. In this study we look into a small selection of the high number of candidate genes that may be associated with anxiety and investigate whether variation in the selected genes could be associated with this undesired behavioral trait.

Abstract

Anxiety is a common problem in dogs and represents a welfare issue to both the dog itself and the owner. Mild to moderate anxiety has a relatively high prevalence in the Havanese, even though a social and outgoing personality is listed as an important breed characteristic. Association between genotype in the dopamine receptor 2-gene (DRD2) and the dopamine transporter-gene (DAT, SLC6A3) has been detected in various species. In this study we investigated genetic variation in DRD2 and DAT of the Havanese and possible association with anxiety phenotype. Phenotype was classified through observation and owner questionnaires.

In this study, we found that:

1.   There is a large degree of heterogeneity in the dopamine transporter gene (DAT) and the dopamine receptor gene (DRD2) of the Havanese.

2.   There is significant association between haplotypes in exon 2 of the DRD2-gene and the level of anxiety in the Havanese, expressed both as owner survey answers and observed phenotype.

3.   There is significant association between haplotypes in exon 10 of the DAT-gene and observed phenotype in the Havanese.

In addition, a mutation associated with a short coat was studied and we also looked into genetic variation for the breed. The results showed that there was a co-segregation of a mutation in the RSPO2-gene and a short coat in the Havanese and the frequency of this mutation was 0,143 in our selection of dogs. Genetic variation was measured as average heterozygosity (0,69) and did not indicate that there is low genetic variation in the Havanese.

Background

Behavioural problems occur frequently in dogs (10) (25) (2). One study (2) showed that anxiety (19,7%), phobias (3,9%) and fears (0,7%) constitute a large portion of problems presented by owners at the Animal Behavioural Clinic at Cornell University. In addition, fear aggression towards owners (5,2%) and strangers (16,8%) were important complaints. The percentage of aggression that was classified as fear aggression was increasing towards the end of the study, indicating that it may have been underdiagnosed early in the study. Behavioural problems are an important cause of both dog abandonment (27) and euthanasia (12), and clearly represent an important welfare issue to the modern pet dog.

In breeding one tries to put emphasis on traits that are both of clinical importance, and have a high heritability. For years, a great amount of work has been put in by researchers, Kennel Clubs and breeders alike, to better the physical health of dogs through breeding.

Health screening animals for breeding, including eye exams and orthopaedic evaluations, are generally well accepted and are considered important tools in dog breeding programs. There is reason to believe though, that behavioural problems are just as important as physical problems to the welfare of dogs.

Being anxious is a welfare issue in itself, as the dog is forced to encounter people, other animals or environments that are scary to them, often on a day to day basis. Anxious dogs might also be subject to secondary welfare issues such as isolation or unethical training methods because of

behavioural problems like barking or aggression. Sometimes anxiety in dogs can result in behavioural problems that are so severe that the dog is surrendered or euthanized.

Anxious dogs can also create a welfare issue for owners if they are unable to use the dog for the activities they intended to, like obedience, agility or hunting. Having an anxious dog can be challenging for the owner because of behavioural problems like separation anxiety or aggression, that make the dog ownership more complicated than expected. Last, but not least, anxiety and behavioural issues in dogs are of relevance to society, as abnormal aggression resulting from anxiety can create dangerous situations for people or other dogs.

Variation in phenotype is a necessity if one is going to improve mentality in dogs through breeding. If there is little genetic variation in a trait – selection will be difficult because no animal is much better than any other as a genetic parent (4). Recognising that there are differences in the level of anxiety expressed by different dogs, will in turn allow us to choose the best dog for breeding.

In a survey conducted by the Norwegian Havanese Club in 2012/2013, 18,6 % of Havanese owners said their dog was "a little nervous" or "very nervous". In a breed where a social and outgoing personality is one of the main breed characteristics listed in the standard, this represents a significant deviation, and indicates phenotypic variation. 31% of owners replied that their Havanese was "a little brave" or "very brave".

Anxiety in Havanese

We have strong evidence that anxiety and other behavioral problems are of great clinical importance, and that there is variation in phenotype within the Havanese breed. To justify prioritizing behavior in a breeding program, we also need evidence that behavior has a high heritability. The different temperament of various dog breeds indicates this. The fact that Border Collies herd and Setters freeze to indicate birds even with minimal training can likely be explained by genetics.

In a study on breed differences (29) five dog breeds (Basenjis, Cocker Spaniels, Shetland Sheepdogs, Fox Terriers and Beagles) were tested in their ability to learn different tasks. Ability to learn and perform these tasks varied between breeds, as well as the dogs' reaction to the challenge.

Several studies have found high heritability's in behavioral traits. Studies have shown moderate to high (>0,40-0,25) heritability for hunting traits like nosework and "pointing" (28). Another study (26) on behavioral traits in dogs estimated the heritability of the shyness/boldness aspect of a dogs personality to be 0,25.

Twin studies of human children have shown that sensitivity to anxiety has an estimated heritability of 0,45 (31). Traits that are associated with Bipolar Disorder, a psychiatric disorder in the anxiety spectrum, were found to have an estimated heritability of 0,53 (girls) and 0,87 (boys) in human children (1). High genetic influence has also been detected for personality traits of shyness, inhibition and fear in people (9).

Anxiety in Havanese

At the University in Arkansas, two lines of pointers were bred; one nervous line and one control line. After five generations the nervous dogs were anxious, timid, inactive and hard to train (22). The control dogs were trainable, social, active and people-seeking. The researchers indicate that the anxiety was a result of additive gene action.

In Russia a selection study was conducted to explain how dogs were originally domesticated from wolves. Foxes in fur farms were tested for tameness and levels of anxiety and aggression. Foxes that showed low levels of anxiety and high levels of tameness and curiosity were mated to each other. After several generations, the foxes started to display friendly and dog-like behavior (32). This can be considered evidence that it is possible to breed away from anxious temperaments, and that anxiety has a high heritability.

Experiments have also been done with mice in a laboratory setting (8). Mice were categorized as fearful or not fearful based on their activity level in an "Open field-box". Three lines were bred; the fearful mice, the not fearful mice, and a control line. After 30 generations the fearful line of mice became increasingly anxious, and the not fearful line of mice became increasingly "brave" and active. The control line showed around the same level of fearfulness as the original mice. This is also strong evidence that "Fearfulness" has a relatively high heritability.

The success of genetic improvement through breeding depends on with what repeatability breeding values can be estimated. Mentality can be more challenging to measure than a lot of physical properties such as conformation or disease. The phenotype may also be altered by environment; which further reduces the accuracy of the EBV. In addition, there will probably be

some disagreement between different breeders on what is a desired temperament, and this will also reduce the repeatability of breeding value estimates.

Another challenge in breeding is of course that prioritizing temperament will have to reduce the emphasis on other properties like conformation and/or health results, unless one increases the selection level and in turn reduce the effective population size, which would be unfortunate.

Research in behavioral genetics could provide new tools to increase efficiency in genetic improvement through breeding. However, researchers face some of the same challenges as breeders in classifying behavior. In quantitative genetics, too much misclassification will easily dilute a small, but significant association to the point where it is undetectable.

In addition to the challenges in categorizing behavioral phenotype that have already been discussed, the large number of dogs that need be classified in a research project provide even more challenges. There are several methods of classification that each has their own advantages and disadvantages.

Using owner questionnaires is a good method for collecting information about a lot of dogs relatively time and cost-efficiently. The effort required from the owner is limited, which is beneficial to recruitment. Surveys also provide information on how the dog functions in everyday life, as opposed to a potentially artificial test situation. The large disadvantage of owner surveys is the repeatability, which will unquestionably suffer when potentially hundreds of different people are doing the evaluation.

An alternative to owner surveys is testing the dogs' behavior in a controlled setting. This will improve repeatability, but it will also be costly and time consuming, especially when a large number of dogs are required for the project. It will also require more effort from the owner, which could influence the number of dogs one was able to recruit. Another factor to take into consideration is that important information can be lost in a controlled test situation. The tests might not be able to detect the specific forms of anxiety that are actually causing problems for the dogs in a real life setting.

One study (34) on heritability of behavioral traits in dogs indicated that a subjective evolution of the dogs' personality, performed by only one or a few experienced people, may be a reliable method for describing complex behavioral patterns in dogs. They also found that only a few criteria were needed in order to describe the behavioral differences between dogs.

Quantitative traits are influenced by a number of loci, each with small effect on phenotype. Even if the hereditability is high, each locus' influence on phenotype might be relatively small. Such loci with small effects are hard to detect, and would require a large sample size. Significant association between markers and phenotypic variation have been detected for behavioral traits, but the percentage of variation explained by each marker is most often low (7).

Loci with significant association to quantitative traits are called quantitative trait loci, QTLs. Such markers are usually not functional, but are in linkage disequilibrium with genes that have a functional additive effect on the trait. As more and more QTLs are identified, they can be

utilized in marker assisted selection. Several QTLs have already been discovered for economically important traits in farm animals.

The preferred method of analysis is association studies. Association studies compare allelic frequencies in groups of high or low scores for quantitative traits, for example anxious and non-anxious individuals. Allelic association studies depend on the alleles to be in linkage disequilibrium with the gene associated with the trait. It is therefore crucial to study parts of the genome that are likely to impact phenotype.

Candidate genes are genes that are suspected to influence a given trait. Candidate genes can be chosen based on the function of the proteins encoded by the gene. For example, because drugs that are commonly used to treat hyperactivity act on the dopamine system, genes that encode dopamine receptors and transporters have often been chosen as candidate genes in association studies on hyperactivity.

Another method of selecting candidate genes is using genes that have been found to be associated with a similar trait, for example in a different breed or species. Both these approaches have been used to select candidate genes for this project.

Pathways and candidate genes

Dopamine, adrenaline, noradrenaline, serotonin, acetylcholine, glutamate and monoamine oxidase are important neurotransmitters in the brain, and are known to influence behavior and mood. Neurotransmitters are chemical compounds that transfer signals from one neuron to

another in the central nervous system. The neurotransmitters are released from the terminal ending of one neuron and binds to the next neuron in what is called a synapse.

Regulation of the amount, release and reuptake/termination of these neurotransmitters is crucial for optimal neurological and mental function. Dopamine is an important neurotransmitter in the amygdala; a part of the CNS that is believed to impact emotion. Research has shown that dopamine levels in the amygdala can influence a person's general anxiety level (14).

Receptors and transporters play important roles in regulating the levels of different neurotransmitters in the brain. Each neurotransmitter is regulated by (usually) a high number of different receptors and transmitters that work together in complex interaction. Each of the receptors are encoded by specific genes, and genetic variation in these genes are important for the function of the receptor and "success of" neurotransmission in the synapsis.

The dopamine neuron has a presynaptic transporter called DAT, which is important to dopamine reuptake, and thereby termination of the nerve signaling. Receptors are also important in the regulation of dopamine concentrations. DRD2 is one of several dopamine receptors. It is prevalent presynaptic, and function as an autoreceptor to ensure negative feedback when dopamine levels are elevated. (30).

In humans, low rate of dopamine reuptake is associated with increased anxiety and irritability (16). DAT-density influence how far into the extracellular space of the striatum dopamine is spread (24). A fragment of variable number of DNA repeats is detected in the 3'UTR of exon 15

in the DAT-gene in humans (33). 98% of the population has either 9 or 10 repeats. People with 9 repeats as opposed to 10, have a 25% lower density of DAT (15).

DRD2 is important in regulation of dopamine levels in the striatum. A polymorphism has been detected in the 3'UTR-region of this gene. The A1-allele is associated with a reduced dopamine receptor density. (15)

A study from 2008 (15) investigated whether individuals with the above mentioned mutations had a lower threshold to anxiety and stress. Individuals were categorized in a 9+ and a 9- group for the presence (9/9, 9/10) or absence (10/10) of the 9 repeat VNTR in the DAT-gene. They were also categorized in an A1+ and A1- group based on the presence (A1/A1, A1/A2) or absence (A2/A2) of the A1-allele in the DRD2-gene. Their level of anxiety was measured using questionnaires.

The study showed that subjects with a A1+9+ genotype displayed significantly higher levels of anxiety than subjects with A1+9-, A1-9+ or A1-9- genotypes. Low density of dopamine transporters causes dopamine diffusion areas to increase. Low density of the dopamine receptors causes less effective negative feedback and dopamine reuptake. It appears that having risk alleles in both the DAT and DRD2-gene, causing a too low density of transporters and receptors alike, result in elevated basal concentrations of dopamine in the striatum. The authors believe the high dopamine concentration could explain the A1+9+ individuals' increased levels of anxiety.
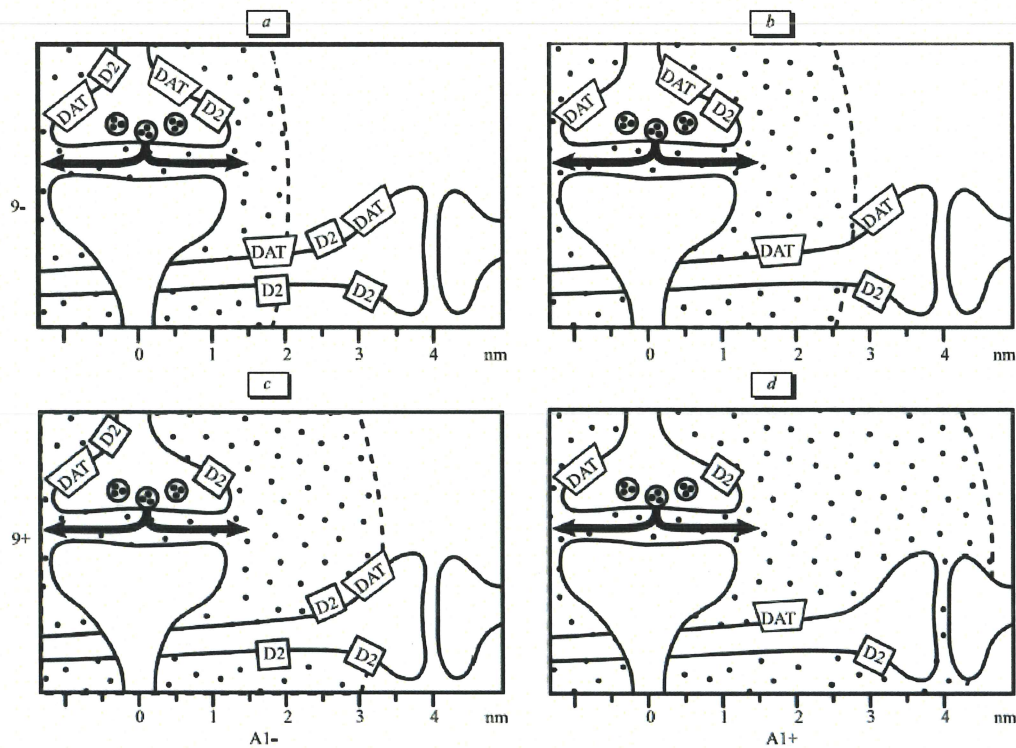
Figure 1: transporter and receptor density, as well as basal dopamine concentrations, in the different genotype groups. The 9+A1+ individuals, *d*, showed increased levels of anxiety. (15).

In a study from 2007 (11), variation in 4 candidate genes (tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH) dopamine transporter (DAT), and dopamine D4 receptor (DRD4)) was studied in 4 breeds; Belgian Malinois (N=50), German Shepherds (N=240), Belgian Tervueren (N=102), Groenandael (N=105) and European Grey Wolves (N=22). The aim of the study was to investigate potential associations of VNTRs in the dopaminergic neurotransmitter system with behavior.

The regions of the genome that were studied were intron 4 of the tyrosine hydroxylase (TH) – gene, intron 4 and exon 11 of the dopamine β-hydroxylase (DBH) –gene, intron 5 and intron 9 of

the dopamine transporter (DAT) –gene, and the previously reported polymorphisms (23) in exon 1 and exon 3 of the dopamine D4 receptor (DRD4) –gene. These regions were chosen because they included VNTRs that were over 6bp long and had more than 85% similarity between the repeated units. Variation was found in all regions except exon 11 of the DBH-gene and intron 5 of the DAT-gene. These were therefore not investigated further.

The previously discovered VNTRs of the DRD4-gene were found in this study as well. The VNTR in exon 3 were polymorphic in all four breeds and the wolves, although the allele frequencies varied among breeds. In addition to the previously discovered alleles, a new allele was found. This allele was longer than the others, presumably because of a 39 base pair insertion.

In exon 1 all known alleles were detected in the dogs, but only one variant were found in the wolves. The genotype frequencies of this VNTR were highly variable among breeds.

The DBH-, DAT- and TH-gene were also investigated. Polymorphism was found in all breeds and the wolves for both the DBH- and the TH-gene. There was some variation in the allele frequencies between breeds.

In the 38 base pair repetitive sequence of intron 9 of the DAT-gene, an interesting discovery was made. All German Shepherds possessed a long variant. This was by far the most common allele in other breeds as well. In the Malinois though, the long and the short variant appeared with

equal frequencies. This sparked interest among other researchers, and the region was later investigated further (see other articles below).

Due to the large number of dogs available, and high genetic heterogeneity, the Tervurens were chosen for further association studies. A classification of activity-impulsivity, (with some similarities with a questionnaire used for ADHD in humans) was performed by the owner. The aim was to investigate whether any of the gene variants were associated with the adapted activity-impulsivity scale symptoms in the dogs, but no association was found.

However, an association was discovered between genotype groups and the attention-deficit score of the dogs. Individuals with at least one DBH allele 1, at least one DAT allele 1, or dogs that were homozygote for the short variant in the DRD4 exon 1, were shown to display a higher degree of attention deficit.

Three other studies look further into variations in the DAT-gene of dogs and different mutations' effect on behavior. The first article (18) studied a previously reported (11) 38 base pair VNTR in intron 9, and the allele frequencies of this polymorphism in different dog breeds. They further investigated whether a one-tandem repeat allele in this location, that is overrepresented in the Malinois, could be associated with various behavioral traits, and even effects on interactions between dog and owner.

The article confirms that the short version of the intron 9 polymorphism is overrepresented in the Malinois breed, as previously described (11). The study investigates not only associations of the

polymorphism with aggression and anxiety, but also associations with epilepsy-like symptoms, such as seizures and lack of responsiveness. They found that dogs that were homozygote for the short/one repeat allele (1/1) were more likely to have seizures, "eyes glazing over", loss of responsiveness and sudden bursts of aggression with no apparent trigger, than dogs that were heterozygote (1/2) or homozygote (2/2) for the long version. No effect of genotype on impulsivity was found. 1/1 dogs were found to be more attentive than 1/2 and 2/2 dogs. This is contradictory to previous results (11).

The study also involved military dogs, and the possible association between the DAT VNTR polymorphism and behavior. No association between aggression levels and genotype were detected. It was however detected that dogs with the 1/1 genotype showed more signals of stress, such as a low body posture and frequent yawning.

A very interesting finding was that dogs with at least one short allele reacted to aversive stimuli with increased signs of stress and decreased performance, compared to dogs with the 2/2 genotype. At the same time handlers of 1/1 and 1/2-genotype dogs were more likely to use aversive stimuli in training than handlers of 2/2 dogs.

It appears that Malinois with the 1/1 genotype, and possibly also the 1/2 genotype, generally have increased levels of stress and anxiety, and even epilepsy-like symptoms to some degree. Their handlers are more likely to use aversive stimuli in training, even if it is proven contra-productive in these dogs.

In another study (19) the DAT-gene was studied in 20 Belgian Malinois and one Belgian Tervueren. 122 polymorphisms were found, including five deletions and 117 SNPs. Six exonic variants that had not previously been reported were detected (one missense and five synonymous). These results indicate that there is great heterogeneity in the DAT-gene of dogs, and that this gene's potential influence on behavioral traits may be complex.

A third article on the DAT-gene in Malinois (20) investigates a different polymorphism; a 22 A VNTR in intron 14. The association between this polymorphism and the activity level and behavior of Belgian Malinois and other breeds were studied. Dogs were evaluated based on owner interviews and activity measurements in a novel and non-novel environment.

234 dogs (165 Malinois and 69 other breeds) were genotyped for this poly(A) insertion. Three different alleles were found. The least common was actually found in the boxer reference sequence (Broad CanFam3.1), and consisted of a 10-nucleotide poly(A) sequence flanked by an 8 base pair target site duplication (GGAAAATC).

The allele found most often in Malinois was similar to the boxer allele mentioned above, except it had 22 A-nucleotides instead of 10. The third and by far most common allele in all other breeds included in the study, was missing both the poly(A) insertion and the (GGAAAATC)-duplication.

```
chr34:11243915

Boxer: GGAAAATC---------------------AAAAAAAAAAGGAAAATC
Other breeds: GGAAAATC ------------------------------------------------------  g.34805del(18)
Malinois: GGAAAATCAAAAAAAAAAAAAAAAAAAAAAAAGGAAAATC  g.34805A[12]
```
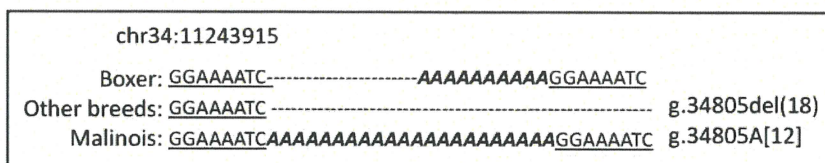
Figure 2: Three alleles detected in intron 14 of the DAT-gene (20)

Owners of Malinois were asked if their dog ever had episodes of seizures, "eyes glazing over", loss of responsiveness or sudden bursts of aggression, without any apparent trigger. Owners of dogs with two polyA(22) alleles were more likely to respond that the dog have had at least one of these behaviors. Owners of dogs with no polyA(22) alleles were more likely to report no issues.

Dogs that were heterozygote had intermediate results. Dogs that have had episodes of "eyes glazing over", but none of the other problems, were most likely to be heterozygote. Dogs that have had both episodes of eyes glazing over and sudden episodes of aggression were more likely to carry at least one polyA(22) allele.

The researchers initially wanted to investigate whether genotype would influence activity level in a controlled test situation. This was somewhat unsuccessful because Malinois in general were more active than other breeds, in addition to the polyA(22) allele being much more prominent in this breed. This could cause the association between genotype and activity level to appear much stronger than it really is. There were also not enough dogs of each breed to study the two groups, Malinois and "Others", separately (only a portion of the genotyped dogs were tested for activity level).

Background – Genetic variation

Havanese were originally owned by Cuban aristocracy. After the Cuban revolution in the 50's

the Havanese was supposedly near extinct. A few Havanese were brought to Florida and Costa

Rica, and it is said that most Havanese decent from these dogs. Dorothy Goodale purchased 6

pedigreed Havanese from two displaced Cuban families. This purchase was comprised of an

adult female, 4 daughters, and an unrelated male. Through an advertisement in a Miami

newspaper, Goodale was later able to purchase a couple more unrelated dogs from an elderly

Cuban gentleman living in Costa Rica.

If most Havanese today decent from these dogs, one could suspect low levels of genetic variation

due to bottle neck effects. For this reason we wanted to calculate the level of the genetic

variation in the breed.

Background – "Short coat gene"

Some Havanese dogs are born with a short coat, which is an undesired conformational trait

according to the breed standard. These dogs also shed, unlike other Havanese. Short haired

Havanese are not common. In most cases when they do occur, a few short haired puppies are

born in litters of otherwise "Normal coated" puppies (personal observation).

Anxiety in Havanese

Picture 1: A short haired Havanese (35)

A lot of uncertainty on the subject has caused different theories to arise, both on the mode of inheritance and even possible co-occurring unfavorable traits. In the 90's there were rumors that the short haired puppies had bad temperaments, but this theory has long been dismissed (37). In later years, a few breeders have started to genotype their dogs with laboratories online, but no one really knows what test is the correct test to use in the Havanese breed. In a survey done on "Short hair" by The Swedish Havanese and Bolognese Club, BBHC, in 2013, one person replied:

"It turns out I did the wrong test, and now I have no idea if the results are correct?!!"

The article "Coat variation in the Domestic Dog is Governed by Variants in Three Genes" (5) describes a GWAS study on coat variation in 80 different dog breeds. More than 1000 dogs were included in the study. They studied mutations in three genes; RSPO2, FGF5 and KRT71, and found that variation in these genes determines most coat types.

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Anxiety in Havanese

In the FGF5-gene a missense mutation in exon 1 is associated with a long coat in some breeds. The short coat variant has a dominant effect. In the RSPO2-gene a 167 base pair insertion is associated with long furnishings. The long furnishings variant has a dominant effect. In the KRT71-gene a missense mutation causes a curly coat. The non-curly variant is dominant.

The study showed that when a dog carries the wild type variant in all three genes, the dog has a short coat. A mutation in the FGF5-gene, combined with the "wild type" allele in the other two genes, causes the dog to have a medium long coat, like the coat of spaniels, retrievers and setters. A mutation in the RSPO2-gene, combined with the "wild type" allele in the other two genes, causes the dog to have a wiry coat, like the coat found in terrier breeds. No dogs with a mutation in the KRT71-gene combined with "wild type" alleles in the two other genes were found. Dogs that have the mutation in the KRT71-gene, combined with mutations in the two other genes, have long and curly coats. Dogs that have mutations in both the FGF5- and the RSPO2-gene have long soft coats, like the Havanese.

	PHENOTYPE	FGF5	RSPO2	KRT71
A	Short	-	-	-
B	Wire	-	+	-
C	Wire and Curly	-	+	+
D	Long	+	-	-
E	Long with Furnishings	+	+	-
F	Curly	+	-	+
G	Curly with Furnishings	+	+	+

A Basset Hound B Australian Terrier C Airedale Terrier
D Golden Retriever E Bearded Collie
F Irish Water Spaniel G Bichon Frise

Figure 3: How mutations in the three genes combine to produce various coat types. (5)

12 Havanese were included in the study. All of them had the long soft coat described in the breed standard. All were homozygote for the long variant in the FGF5-gene. One was heterozygote in the RSPO2-gene and all the others were homozygote for the long furnishings-variant. 3 Havanese were heterozygote for the KRT71-gene, eight were homozygote for the non-curly allele, and one did not have a known genotype for this gene.

Aims of the study

The aims of this study were to:

- Select a few candidate genes for anxiety studies in the Havanese breed.

- Investigate if there is heterogeneity in these candidate genes in the Havanese breed.

- Investigate if an association can be detected between genotype groups and an anxious phenotype in the Havanese breed.

In order to recruit as many dogs as possible for the study, we also wanted to create a DNA-test for the improper short coat in Havanese, which could be offered to owners free of charge if they donated samples for the anxiety study. Secondary aims were therefore to:

- Create a DNA-test for the improper short coat in Havanese.

- Investigate the prevalence and inheritance pattern of this trait in the Havanese breed.

In addition;

- Study genetic variation in the breed using molecular markers.

Methods

Selection of candidate genes for anxiety in Havanese

A thorough literature study (17) revealed a number of potential candidate genes associated with general anxiety (GAD), which is characteristic of anxiety in the Havanese. DRD2 (Dopamin receptor D2) and DAT (dopamine transporter, SLC6A3) have been shown to be associated with generally higher levels of anxiety in people (15) and were chosen as candidate genes for this study.

Selection of candidate genes –"Short hair gene"

A large study (5) on coat variation in the domestic dog revealed that two genes; FGF5 and RSPO2 are important in determining coat length. Because of the suspected mode of inheritance of short coat phenotype in Havanese (autosomal recessive), RSPO2 was chosen as a probable candidate gene for this study.

Primers

Primers embracing all exons and UTRs were created using Primer3plus. Amplification was successful for all parts except for exon 1 in DRD2, exon 12 of DAT, and parts of the 3'UTRs in both genes. Optimal temperatures were detected using a temperature gradient PCR-program with temperatures ranging from 54 to 64 degrees Celsius.

Primers are listed in the table 1.

Table 1: Primers used this study

DRD2	Forward	Reverse	Optimal temperature
1	CGGACGGCTGCCAGG	CGGACAAACTTGTGGTCCCA	No product
2	CCGGTGGTTGATTTCAGCTC	GCAACTTGTTGGCAGGAACC	57
3	GGAAGGAGAGCCCCGCTATA	ATGCACGCACAAACACATGG	62
4	AAGGCACAAGGTGTCTCTGG	CGGCCTCAGTCCCTATCTCT	59
5	GCGTACTCTGTCACATGGCT	CCACCCATCACAGGCCAG	63
6	CTTCACTCTTGCCTCCCCTG	GTGCCTGCTTGTGACTTGTG	58
7	ACCCGGTGAGGCTGAGTG	GAAGGGGATGGCAGGTAAGG	58
8-1	GCCCGTAGCACCCAATCTT	TAGCACTACCCCGGCAGAT	58
8-2	CGGACCAGGCCTTCTCTTTG	CTTCTCTGGGGTTCAGCCTG	No product
8-3	GGTGGGGATGGACAGTTCAC	AGTGGTTTTGTGGCAGGAGG	62
8-4	TCGTAGCAATTGTTGGGCCT	GGGTCACCCTTTCTTGGAGG	No product
DAT			
1	CGCGGTTCAGGCTGCTAA	GCGTTAGGAGCTCCGTCTC	62
2	GACCTGTTTCCCTCTCGGTG	TTCGCCATCGCATCTCAAGT	62
3	AGGTCAGTCCACAGGGTTCT	CAGCAGGCAGTCCGTTTAGA	62
4	TGAATTTTGCCACCTGAGCC	CACCAGACAAGGGACCTCAC	63
5	CTGCCCTGCGTCTTAGCTAG	CTCCGTCCGCACCCCTAC	61
6	CTGAGCTGGCATCCCATGAA	AGAGCAACCCATACCTGCTG	62
7	ATCCCCCAAGACAGCCTTTG	GCAAGAACCAACATGGCCTG	58
8	GAACGCATCCTCCAAAACCC	AACACCTGCAACCTTGGTGA	59
9	TCAGAGTTCCCCTTCCTGGA	CAGGCACGGGAAGTCTGG	62
10	TGACACCCCATCATGACGAC	CATGTGGCTCTCAGGCTCG	61
11	AGAAGGCCTGTCCCTGAGAA	GGTTTAAGCATCCTGGGGCT	60
12	CACCCCTGCAGGACATGG	CTCCCCTGCAGATGCTGAC	No product
13	CCCCCAGACACGGTAATGTG	AGAAACAAAGGGGCAGCGAC	63
14	CCTTACTCAGAGCCCACACG	AATGAAGGCAGCCTCGTGG	63
15-1	ACGGGGCGTATTTGTGGAAG	TCCTCAACCCAGGGATGACA	63
15-2	TCTGTCCGTGTTCCGTTTGT	GTTTTCCTCCCCAGGTCCC	No product
15-3	CTACGGGAGGAAGGGCCG	ACAAACTCGTGTCCCCAGTG	63
15-4	CACTGAGCGGTCACAGCG	GTCGAGGTTAGGCCCAGTAC	62

A study (20) revealed that a poly(A) insertion, primarily found in Belgian Malinois, is associated with behavioral changes. This poly(A) insertion is located in the intron between the 14th and 15th

exon. The primers F: CAGATCAGACATTACTCTAACTATTGC and R:

TCATCAAGCAGGGAAAAAGG (20) were also used in this study.

Primers – "Short hair gene"

The primers used in the Cadieu study (5) (F: AAATTACCATCATGAGACCATGC, R:

TGGCTAAAGAAAACTTCCACAA) were obtained. Two microsatellites were detected in

proximity to the insertion described by Cadieu et al. One, "MS1", is located in intron 5 (F:

CTGTTGTGGCCAGGAAATGC, R: CTGCTACAGCCAGGCTCTTT). The other, "MS2" is

located immediately after the 3'UTR (F: GTGATCCTGGAGACCTGGGA, R:

TCCTTACAAGCTGCGTAGGT).

Dilution of primers

Primers initially had a concentration of 200 pmol/µL, and were diluted to a concentration of

5pmol/µL. 5µL of the primer was added to an Eppendorf tube. 195 µL of distilled water was

then added to the tube, and the sample was vortexed for a few seconds.

DNA extraction

DNA-samples were collected from a total of 188 dogs; 102 samples were EDTA-blood collected

by the author, and 86 samples were buccal swabs, mainly collected by the owners.

For the EDTA-blood the following protocol was used for DNA-extraction:

1. 100 µL EDTA-blood was added to an Eppendorf tube. The rest of the EDTA-blood was
   stored at -20 degrees Celsius.

2. 1mL of refrigerated Lysis buffer was added to the tube.

3. The sample was vortexed for a few seconds

4. The sample was centrifuged for 1 minute at 13000 o.p.m

5. The supernatant was poured off

6. Steps 2-5 were repeated

7. 1 mL of refrigerated TE buffer was added to the sample

8. The sample was vortexed for a few seconds

9. The sample was centrifuged for 2 minutes at 13000 o.p.m

10. The supernatant was poured off.

11. The DNA-pellet was resuspendated in 100 µL PCR buffer with Proteinase K (10µl Proteinase K/1ml buffer)

12. The sample was thermomixed with shaking at 56 degrees Celsius for two hours

13. The sample was thermomixed at 95 degrees Celsius for 10 minutes to inactivate the Proteinase K.

A couple of the samples were coagulated or dry and therefore harder to get a good extraction from. For these samples Kit-isolation with a E.Z.N.A® Blood DNA Mini Kit by Omega Bio-Tek was used:

1. DNA Wash Buffer was prepared by adding 100% ethanol. Dilute HBC Buffer was prepared by adding isopropanol.

2. The Elution Buffer was heated to 65 degrees Celsius.

3. 250 µL of EDTA-blood was added to an Eppendorf tube.

4. 25 µL OB Protease Solution and 250 µL BL Buffer was added to the sample.

5. The sample was vortexed for 15 seconds.

6. The sample was incubated at 65 degrees Celsius for 10 minutes, and vortexed briefly once during incubation.

7. 260 µL of 100% ethanol was added to the sample.

8. The sample was vortexed for a few seconds, and then centrifuged briefly to collect any drops from the lid.

9. A HiBind®DNA Mini Column was inserted into a 2mL Collection Tube.

10. The entire sample was transferred to the column.

11. The sample was centrifuged at 10000 x g for 1 minute

12. Both the filtrate and the collection tube were discarded.

13. The HiBind® DNA Mini Column was inserted into a new 2mL Collection Tube.

14. 500 µL of HBC Buffer (prepared with isopropanol) was added to the sample.

15. The sample was centrifuged at 10000 x g for 1 minute.

16. The filtrate was discarded and the Collection Tube was reused.

17. 700 µL of DNA Wash Buffer (prepared with ethanol) was added to the sample.

18. The sample was centrifuged at 10000 x g for 1 minute.

19. The filtrate was discarded and the Collection Tube was reused.

20. Steps 17-19 were repeated.

21. The empty HiBind® DNA Mini Column was centrifuged at maximum speed for 2 minutes to dry the column.

22. The HiBind® DNA Mini Column was inserted into an Eppendorf Tube.

23. 100 µL of (heated) Elution Buffer was added to the column.

24. The sample was incubated at 65 degrees Celsius for 5 minutes.

25. The sample was centrifuged at 13000 x g for 1 minute.

26. Steps 23-25 were repeated.

Anxiety in Havanese

DNA isolation form buccal swabs:

Swabs by Performagene™ from the PG-100 collection kit were used. The owners were advised to collect the sample when the dog had not eaten or been for a walk for a few hours. They were also instructed to avoid contamination of the swab, and to properly rub the swab against the dog's gums to ensure enough DNA was obtained.

For the buccal swabs the following protocol was used for DNA isolation:

1. The sample was manually shaken for a few seconds.

2. The sample was incubated in the thermomixer at 50 °C for a minimum of one hour.

3. The cap was removed and the collection sponge was pressed against the inside of the tube to extract as much of the sample as possible.

4. The sponge was cut of the cap with scissors and discarded.

5. 500 μL of the mixed Performagene sample was transferred to an Eppendorf tube. The cap was put back on the original tube and the rest of the sample was stored at -20 degrees Celsius.

6. 20 μL of PG-L2P purifier was added to the sample and the sample was vortexed for a few seconds.

7. The sample was incubated on ice for 10 minutes.

8. The sample was centrifuged for 5 minutes at 15000 x g.

9. The supernatant was carefully transferred to a fresh Eppendorf tube. The pellet and old tube was discarded.

10. 25 μL of 5 M NaCl solution was added to the sample.

11. 600 μL of room temperature 100% ethanol was added to the sample.

12. The sample was gently mixed by inversion 10 times.

13. The sample was then left at room temperature for 10 minutes.

14. The tube was placed in the centrifuge in a known orientation and centrifuged for 2 minutes at 15000 x g.

15. The supernatant was carefully removed with a pipette, without disturbing the DNA pellet.

16. The DNA was washed by adding 250 µL of 70% ethanol.

17. The ethanol was carefully removed after 1 minute.

18. The tube was centrifuged briefly to pool any remaining ethanol so that it could be removed.

19. The DNA pellet was resuspendated in 100 µL TE buffer. Then the sample was vortexed.

20. The sample was left at room temperature overnight (or for a couple of days), and vortexed briefly a couple of times during the incubation.

All DNA was stored at -20 degrees Celsius after isolation.

PCR

The dNTP mix was created by mixing 30 µL of each of the 4 100M nucleotide solutions, dATP, dCTP, dGTP, dTTP (Amplicon) with 1080 µL of distilled water.

14 µL of PCR mix, enough for 1 µL of DNA sample, included the following:

- 10,65 distilled water

- 1,5 µL Standard buffer (Amplicon)

- 0,5 µL dNTP mix (2,5mM) (Amplicon)

- 0,5 µL forward primer (5pmol/µL) (Eurofins Genomics)

- 0,5 µL reverse primer (5pmol/µL) (Eurofins Genomics)

Anxiety in Havanese

- 0,05 µL Taq DNA polymerase (Amplicon)

- 0,3 µL Q-solution (Qiagen)

For each reaction, 1µL of isolated DNA was used. Samples were heated to 95°C for 2 minutes for initial denaturation, followed by 38 cycles of 30 seconds at 95°C, 40 seconds at optimal temperature (se fig. x) at 30 seconds at 72°C, and a final 7 minute extension at 72°C.

Gel electrophoresis

Loading buffer was made by mixing 0,5 mL 1% bromophenol blue, 11,5 µL glycerol and 20mL H20. 5 µL PCR product was mixed with 7 µL loading buffer and analyzed by agarose gel electrophoresis on a 1% Lonza SeaKem® LE Agarose gel with ethidium bromide. The samples were run at 200V for 40 minutes. The samples were visualized under UV light using a Syngene Gene Genius Bio Imaging System and Genesnap program. The objective of this was to check for a PCR-product before sequencing.

Gel electrophoresis – "Short hair gene"

Gel electrophoresis was used diagnostically for the RSPO2 gene. The mutation associated with coat phenotype is a 142 base pair insertion, which meant the different genotypes could be visualized on the agarose gel.

Sequencing

PCR-products were sequenced using a Big Dye 3.1 kit (Applied Biosystems). For each reaction the following mix was used:

- 1 µL PCR product

- 2 µL refrigerated Big Dye buffer

- 1,5 µL Big Dye terminator mix from freezer

- 0,5 µL Forward or reverse primer

- 5 µL distilled water

Samples were heated to 95°C for 1 minute, followed by 29 cycles of 96°C for 15 seconds, 50°C for 10 seconds and 60°C for 2 minutes.

Samples were then subject to a precipitation reaction, using the following procedure:

- Samples were transferred to a microtiter plate for ABI 3100

- 2,5 µL 0,125 M EDTA were added to each sample

- 30 µL of 96% ethanol were added to each sample, and the samples were mixed with the pipette.

- The samples were incubated for 15 minutes at room temperature

- The samples were centrifuged at 4000 rpm for 30 minutes

- Immediately after centrifugation the tray with samples were turned upside down on a tissue to remove the liquid.

- The tray was then centrifuged upside down at 350 rpm for 1 minute.

- 60 µL of 70% ethanol was added to each sample.

- The samples were centrifuged at 4000 rpm for 10 minutes.

- Immediately after centrifugation the tray with samples were turned upside down on a tissue to remove the liquid.

- The precipitate was mixed with 12 µL of distilled water.

The samples were then sequenced using Applied Biosystems 3500 xL Genetic analyzer.

Sequences were analyzed using a Sequencher 5.1 program.

Animals

DNA was collected from 188 Havanese; 102 EDTA blood samples and 86 buccal swabs. Blood samples were originally collected at dog shows, but this practice was soon discontinued because some owners feared that blood sampling at the show would negatively influence show performance in an anxious dog. To avoid selection error, blood sampling at dog shows was substituted for home visits at breeders and owners in various parts of Norway. Owners were also welcomed to give blood samples at our lab. Buccal swabs sent via mail were used when blood sampling was impossible due to geographic distance. Some buccal swab samples were collected at "Havanese play groups" in a local park.

Dogs were recruited through different channels. The project was promoted through the breed clubs webpage www.nbhk.info, the breed clubs newsletter "Havanaisern", the breed clubs Facebook page, and other Norwegian Havanese Facebook groups. Breeders were also contacted via e-mail and phone. The project was promoted at dog shows.

All Norwegian Havanese were invited to participate, regardless of phenotype. Two of the dogs were specifically recruited because they had short coats, and their DNA was needed for the development of the RSPO2-test. The rest of the dogs (n=186) were recruited randomly from the population, although anxious Havanese were specifically encouraged to participate. The DNA-test for the short coat was given free of charge to everyone who donated DNA samples to the project, in an attempt to attract more participants.

All major Havanese breeders in Norway are represented by at least a few dogs. Dogs from over 60 different kennels have participated.

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The anxiety study

All exons were initially sequenced for a small group of 8 unrelated dogs to identify regions with variation. Exons where there was no variation were not investigated further. Exons with variation were then sequenced for a test group of 11 cases and 13 controls. When indication of association was detected, the region was later genotyped for all (n=188) individuals.

Behavioral classification -cases and controls

Dogs were classified as cases or controls by two different methods; through an owner questionnaire and through a systematic observational approach. These two scores were later combined in an overall score. A behavior questionnaire (Survey Classification) was sent out to all dogs that had donated DNA-samples to the project, and the aim was to give a quantitative measurement of the level of anxiety the dogs are displaying. The multiple questions gave us the opportunity to study individual aspects of behavior, as well as different combinations of anxiety-associated variables. Additionally we also did an Observational Classification performed by the same person (KB). The aim of the Observational Classification was to obtain an independent classification that was less influenced by the owner compared to the Survey-based classification. The Overall Score sought to combine the qualities of the two other means of classification, and was calculated as an average of the two other scores when both were available.

Owner questionnaire

The questionnaire consisted of 31 questions. The first part (question 10-17) consisted of questions regarding environmental factors that might influence anxiety, so that we would be able to correct for such factors in the genetic analysis. The second part of the study (19-30) comprised questions on behavior. The owners were asked to agree or disagree with various statements on the dogs' behavior. Answers were given on a five point scale (ranging from strongly agree to strongly disagree). Depending on the statement, the answers were then given points between 1

and 5, with 1 representing a high level of anxiety and 5 representing a low level of anxiety. All individuals were then given a "Survey Score" which was the average of answers to questions 19-29. Owners of 150 dogs responded to the questionnaire.

Observation

The second approach was observational. At the time of DNA-sampling the dog was observed, and based of four criteria the dog was subjectively classified. Only dogs that showed a clear majority ($\geq$ 3) of either case- or control-criteria were classified accordingly.

Table 2: Criteria for observed phenotype classification

	Anxious	Control
First contact with external observer	Pulling away	Actively contact seeking
Tail position	Down	Up
Reaction to gentle restraint	Strong avoidance	No avoidance, or positive reaction
Owner statement	Without being prompted the owner mentioned that the dog is anxious.	Owner does not indicate that the dog is anxious

When the observation was inconclusive (e.g. the dog only displays *some* of the criteria or a mixture of case- and control-criteria) the dog was not included in the observed phenotype association studies. An observational classification was also not available for dogs whose DNA-sample was sent via mail. A total of 109 dogs obtained an observational classification. 32 were

evaluated as cases and 33 were evaluated as controls. 44 were inconclusive, and therefore excluded.

Overall score

The third classification was done by combining the average survey score and the observational classification. The aim was to improve the accuracy of the phenotypic estimate, and to include as many dogs as possible. First, all dogs that lacked both an Observational Classification and a Survey Score (n=31) were excluded. Then the dogs' "Survey Score" was corrected with data from their "Observational classification", to create an "Overall Score". Note that for some dogs both an observational and a survey based classification is available, whereas others only have been classified through either the questionnaire, or through observation. The overall score should therefore be considered a "rough estimate" of the general anxiety level of the dog.

Overall score (when both a survey score and an observational score was availible)

$$= \frac{Survey\ Score\ (range\ 1-5) + Observational\ Score\ (Anxious = 1, Inconclusive = 3, Control = 5)}{2}$$

Phenotypic information

150 owners answered the questionnaire, and observational classification was available for 65 dogs. 58 dogs had both a survey score and an observational score. The table illustrates how many dogs were classified in each method of classification. Note that many dogs have both a survey score and observed phenotype, and this is the reason the numbers add up to more than the total number of dogs.

Table 3: The number of Havanese classified with each method of classification.

Owner questionnaire	n = 150
Observational classification	n = 65 (excluding "inconclusive" phenotypes)
Overall score	n = 157

Questionnaire

Both observational and survey based classification

150

58   65

Observational classification
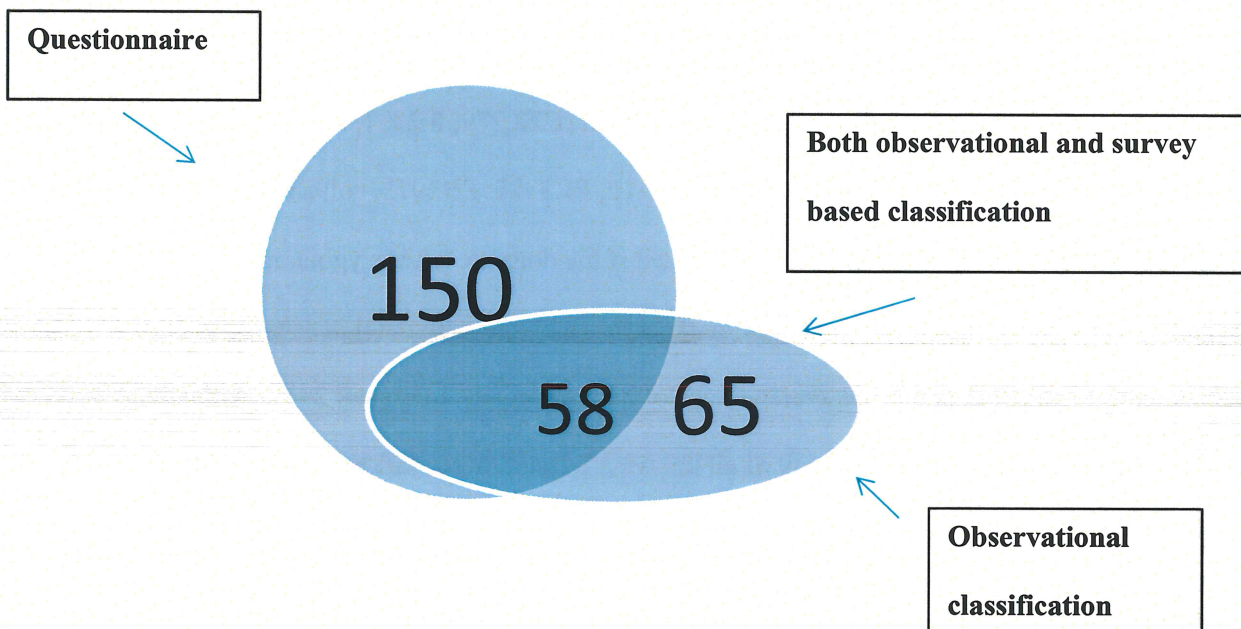
Figure 4: Number of dogs that are classified with the different methods of classification.

The "Short Hair Study"

Based on literature studies the RSPO2 gene was selected as a candidate gene. Two Havanese

with a short coat, and three known carriers were initially genotyped. Later, relatives of these

dogs were also genotyped, and pedigree analyses were conducted to give the study more power.

All Havanese (n=188) were then tested for the RSPO2 polymorphisms. Pedigree analysis were

conducted for these Havanese as well, to make sure the gene followed a simple Mendelian inheritance.

Genetic heterozygosity

A random sample of 21 Havanese was selected using the "Random number function" in Excel. All individuals with a number over 0,8 were selected (=20% of the dogs). When full siblings or parent-offspring were both pulled, one of them was removed from the selection.

The dogs were genotyped for the following markers: Aht211, C22.279, fh2001, FK2054, FH2247, FH2289, FH2293, FH2328, inra21, PEZ8, pez12, FH3325, FH2973, col9a3, cfa5-61tttc21, rdh5 taga 10, cfa-fh3813, fh2772. It was noted if the dogs are heterozygote or homozygote at each marker. The percentage of markers in which the individual is heterozygote was calculated for each dog. Later, the average level of heterozygosity among all 21 individuals was calculated. It was also noted how many of all known alleles at these markers were found within the sample.

Statistical analyses

Odds ratios and P-values were calculated using 2x2-tables. The calculations were done manually and with © 2015 MedCalc Software.

Results

SNPs identified in DAT and DRD2

8 unrelated Havanese were sequenced for all exons in the DRD2- and DAT-gene to look for

variation. 8 SNPs were identified in the drd2-gene. 3 are located in introns, 3 are located in exons

and 2 are located in the 3'UTR. The three exonic SNPs are synonymous. None of these

polymorphisms are previously reported.

27 SNPs were identified in the DAT-gene. 18 are located in introns, 7 are located in exons and 2

are located in the 3'UTR. 5 of the exonic SNPs are synonymous. In exon 2, two missense

mutations (c/g and c/t) are located next to each other (complete LD), causing an amino acid

change from proline (CCC) to valine (GTC).

10 (5 intronic and 5 exonic) of the SNPs in the DAT-gene have not been previously reported.

Table 4: Location and nature of the SNPs, and whether they are previously reported or not. SNPs
that appeared to be associated with behavior-phenotype in the test group are highlighted.

Index	Gene	Intron/exon	Location	Alleles	Type	Annotated in CanFam3.1	Annotated by Lit et al. (ref)
1	DRD2	Intron 1	5:19782497	g/a	Intron	No	-
2	DRD2	Exon 2	5:19782667	c/t	Synonymous	No	-
3	DRD2	Exon 2	5:19782829	c/t	Synonymous	No	-
4	DRD2	Intron 4	5:19787766	c/t	Intron	No	-

5	DRD2	Intron 4	5:19787788	c/t	Intron	No	-
6	DRD2	Exon 7	5:19791794	c/t	Synonymous	No	-
7	DRD2	Exon 8	5:19794262	g/a	3'UTR	No	-
8	DRD2	Exon 8	5:19794287	c/t	3'UTR	No	-
9	DAT	Intron 1	34:11211260	c/a	Intron	No	Yes
10	DAT	Intron 1	34:11211282	g/a	Intron	No	Yes
11	DAT	Intron 1	34:11211298	g/a	Intron	No	Yes
12	DAT	Intron 1	34:11213676	g/a	Intron	No	No
13	DAT	Exon 2	34:11213702	c/g	Missense,	No	No
14	DAT	Exon 2	34:11213703	c/t	pro → val	No	No
15	DAT	Intron 2	34:11213766	g/a	Intron	No	No
16	DAT	Intron 2	34:11214740	c/t	Intron	No	No
17	DAT	Intron 2	34:11214744	g/a	Intron	No	No
18	DAT	Exon 4	34:11217667	g/a	Synonymous	Yes	Yes
19	DAT	Intron 4	34:11225651	g/a	Intron	No	Yes
20	DAT	Exon 5	34:11225779	g/a	Synonymous	Yes	Yes
21	DAT	Intron 6	34:11226749	c/t	Intron	No	Yes
22	DAT	Intron 6	34:11230518	g/a	Intron	No	Yes
23	DAT	Intron 8	34:11232039	c/t	Intron	No	Yes
24	DAT	Intron 8	34:11234689	c/t	Intron	No	Yes
25	DAT	Intron 9	34:11234873	g/a	Intron	No	Yes
26	DAT	Intron 9	34.11234884	g/a	Intron	No	Yes
27	DAT	Intron 9	34:11235772	g/a	Intron	No	No

28	DAT	Intron 9	34:11235810	c/a	Intron	No	Yes
29	DAT	Exon 10	34:11235835	g/a	Synonymous	Yes	Yes
30	DAT	Exon 11	34:11236709	c/t	Synonymous	Yes	Yes
31	DAT	Exon 11	34.11236718	g/a	Synonymous	No	No
32	DAT	Intron 11	34:11236787	c/t	Intron	No	Yes
33	DAT	Intron 11	34:11236849	g/a	Intron	No	Yes
34	DAT	Exon 15	34:11245650	c/t	3'UTR	No	No
35	DAT	Exon 15	34.11246664	a/c	3'UTR	No	No

Exon 2, 3, 7 and 8 of the DRD2-gene, and 2, 4, 5, 10 and 11 of the DAT-gene were sequenced in a test group of 11 cases and 13 controls to look for indications of association between genotype and phenotype. In the test group there were no indications of association between phenotype and SNPs in exon 3, 7 or 8 in the drd2-gene, or exon 2, 4, 5 or 11 in the DAT-gene.

There were indications of strong association between haplotypes in exon 2 of the drd2-gene and phenotype. Because of this finding exon 2 was typed for all individuals.

There were also indications of association between phenotype and haplotypes in intron 9/exon 10 of the DAT-gene. For this reason, this region was also typed for all individuals.

Intron 4 and 3'UTR of the DRD2-gene were typed for all individuals in order to create haplotypes.

DRD2- haplotypes

183 Havanese were sequenced for exon 2, intron 4 and 3'UTR of the DRD2-gene. Haplotypes were created using manual inspection of the data, and the program Haploview (3). It was not possible to create haplotypes across the whole of DRD2. Haplotypes were therefore created for exon 2 and intron 4/3'UTR separately.

The most common haplotypes in exon 2 were CT (Haplotype 1) and TC (Haplotype 2), and their allele frequencies were 0,623 and 0,359 respectively. Only these haplotypes were used in the further association studies.
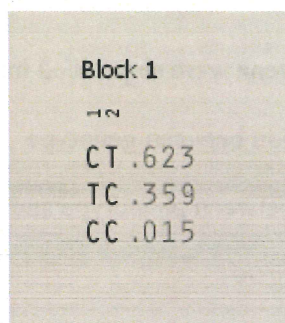
Block 1

CT .623
TC .359
CC .015

Figure 5: Haplotypes in exon 2 of the DRD2-gene

The most common haplotypes in the last part of DRD2 were TCA (Haplotype 1) and CTG (Haplotype 2), and their allele frequencies were 0,667 and 0,206 respectively. There was no indication that these haplotypes were associated with anxiety, and they were not included in the further association studies.
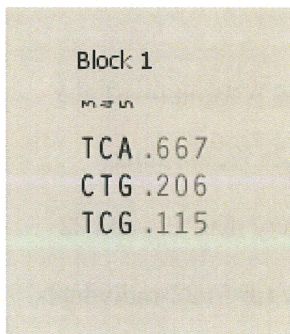
Anxiety in Havanese

Block 1

TCA .667
CTG .206
TCG .115

Figure 6: Haplotypes ranging from intron 4 to 3'UTR in DRD2

Association of DRD2 (exon 2, CFA 5) with behavior

The two SNPs (location 5:19782667 and 5:19782829) in exon 2 lay 162 base pairs apart and are in strong linkage disequilibrium. The linkage phase of the SNPs are cytosine in the first SNP and thymine in the second SNP in haplotype 1, and thymine in the first SNP and cytosine in the second SNP in haplotype 2.

Table 5: Haplotypes in exon 2 of the DRD2-gene

	5:19782667	5:19782829
Haplotype 1 (H1)	C	T
Haplotype 2 (H2)	T	C

Because of the high LD, haplotypes were investigated rather than single SNPs in the association study. Possible association between the two haplotypes and phenotype was studied, using three different phenotypic measures.

Observational classification

Based on systematic observations at the time of DNA-sampling, 32 Havanese were classified as anxious and 33 Havanese were classified as controls.

Dogs that were homozygote for haplotype 1(11) in exon 2 of the drd2-gene were significantly more likely to display anxiety than heterozygote (12) individuals (OR: 14.2857, P-value: 0.0001). 11-individuals were also significantly more likely to display anxiety than 12- and 22- individuals combined (OR: 10.8000, P-value: 0.0001). There were too few (n=5) 22-indivduals to compare the two homozygote groups. The 22-individuals were however evenly distributed among the cases and controls (table 6).

Table 6: Observed phenotype and DRD2-genotypes

	Anxious	Controls
H1H1	20	5
H1H2	7	25
H2H2	3	2
Other haplotypes	2	1
	32	33

Table 7: OR-tables DRD2-gene, observed phenotype

	Anxious	Controls		Anxious	Controls
H1H1	20	5	H1H1	20	5
H1H2	7	25	H1H2+H2H2	10	27

OR: 14,3/P:0,0001   OR:10,8/P:0,0001

Survey based classification

150 owners replied to the questionnaire. The lowest recorded individual survey score was 1,85,

and the highest was 5,0. The average score was 4,07 and the median was 4,28.
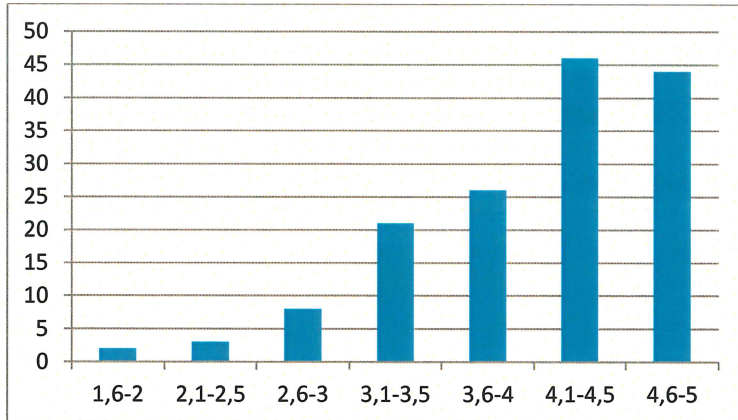

Figure 7: Distribution of survey scores

Due to the quantitative nature of behavior, including anxiety, and the fact that there are no

distinct objective cut-off between cases and controls, different cut-offs were explored.

Regardless of the cut-off, there were always a higher frequency of 11-individuals than 12-

individuals among the cases, and always a higher frequency of 12-individuals than 11-

individuals among the controls.

When the cut off was set close to the median, between 30% and 50%, there was no significant

association between average survey score and DRD2 haplotype (P-value: 0.0896-0.1482). The

calculated odds ratios were however still between 1.9714 and 1.6897, thus supporting an

association between DRD2-genotype and average survey score.

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When the cut off was set higher, so as to compare the most mentally stable individuals to "others", there was significant association between the average survey score and DRD2 genotype. When comparing the 25% of Havanese with the highest survey scores to the rest of the Havanese, 12-individuals were significantly more likely to place in the non-anxious group than 11-individuals (OR: 2.4419, P-value: 0.0410). When the cut-off was increased further, so as to compare the Havanese with the 20% highest scores to the rest, the 12-individuals odds of placing in the control group compared to the odds of the 11-individuals increased even more (OR: 3.2553, P-value: 0.0219).

Table 8: Genotypes DRD2-gene and phenotypes, questionnaire based classification

	Cases	Controls (top 20%)	Cases	Controls (top 25%)
H1H1	54	6	50	10
H1H2	47	17	43	21
H2H2	16	4	16	4
Other haplotypes	4	2	4	2
	121	29	113	37

Table 9: OR-tables with cut-off set at top 20%

20%	Cases	Controls	20%	Cases	Controls
H1H1	54	6	H1H1	54	6
H1H2	47	17	H1H2+H2H2	63	21

OR:3,3/P:0,0219   OR:3,0/P:0,0276

Table 10: OR-tables with cut-off set at top 25%

25%	Cases	Controls	25%	Cases	Controls
H1H1	50	10	H1H1	50	10
H1H2	43	21	H1H2+H2H2	59	25

OR:2,4/P:0,0410   OR:2,1/NS

When comparing 11-individuals to 12-individuals and 22-individuals combined, there is significant association between drd2 genotype and average survey score (OR: 3.000, P-value: 0.0276) with a cut off at the top 20%. The only way to get at least 5 individuals in each cell in the OR-table when comparing 11-individuals with 22-individuals, excluding the heterozygotes, was if the cut off was set at the median. No significant association between the two homozygote groups and average survey score was detected at this level (OR: 1,71, P-value: 0,3018). The 22-individuals appeared with similar frequencies in the case and control groups.

Overall score classification
When an overall score was calculated as previously described, phenotypic information was available for 157 Havanese. Scores ranged from 1,0 to 5,0, with 1 being the most anxious and 5 being the least anxious. The average score was 3,53, and the median was 3,66.
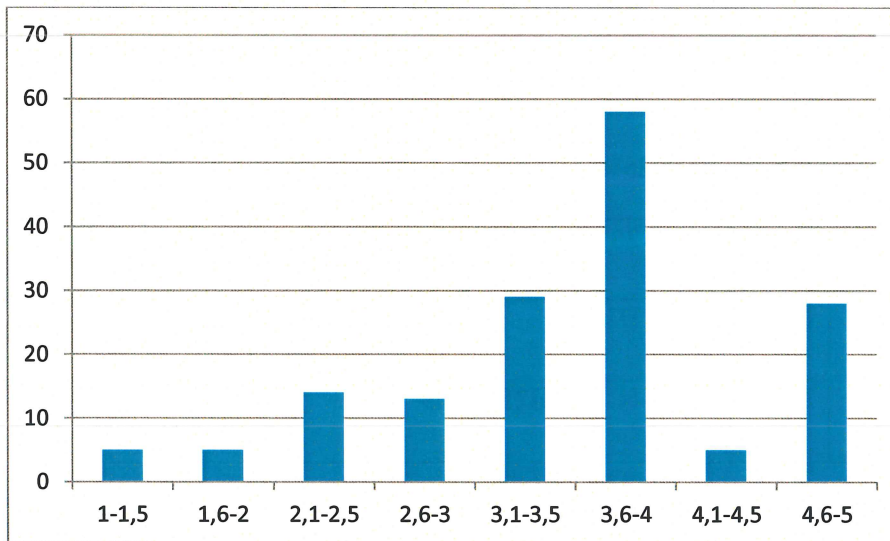
Figure 8: Distribution of overall scores

The 11-individuals were significantly more likely to be anxious than 12-individuals, regardless of at what level the cut off was set between top 20% and bottom 20% (OR: <7,76, P-value: <0,0001-0,0346). The 11-individuals were also significantly more likely to be anxious than the 12- and 22-individuals combined (OR: <6,08, P-value: 0,0001-0,0261).

The OR-values were higher when comparing 11-dogs to 12-dogs, than when comparing 11-dogs to 12- and 22-dogs combined, except at the lowest cut off. The frequency of 22-individuals were highest in the case-group when the cut off was set at the median or higher, and highest in the control group when the cut off was set below the median.

When comparing 11-individuals to 22-individuals, excluding the heterozygotes, there was no significant association between genotype and overall score, and the OR ranged from 1 to 2,5.

Table 11: Odds ratios and P-values from the Overall Score Association Study.

| Controls	Cases	11 vs. 12		11 vs. 12+22	
		Odds ratio	P-value	Odds ratio	P-value
Top 20% N=31	Bottom 80% N=126	6,06	0,0007	4,60	0,0035
Top 30% N=48	Bottom 70% N=109	7,76	<0,0001	6,08	0,0001
Top 40% N=64	Bottom 60% N=93	5,05	<0,0001	3,50	0,0006
Top 50% N=77	Bottom 50% N=80	3,56	0,0006	2,63	0,0045
Top 60% N=94	Bottom 40% N=63	2,76	0,0058	2,65	0,0045
Top 70% N=110	Bottom 30% N=47	2,28	0,0346	2,24	0,0261
Top 80% N=126	Bottom 20% N=31	3,18	0,0130	3,69	0,0051

Average and median of the different genotype groups – DRD2-gene
The average and median of all three genotype groups (11, 12, 22) were calculated for all three

phenotype classifications (observational, survey based and overall score).

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Table 12: Average score for each genotype (11, 12, 22) using three different methods of classification. *(Anxious=0, Control=1)

	Observational*	Survey	Overall
Average 11	0,2	3,98	3,21
Median 11	-	4,15	3,37
Average 12	0,78	4,11	3,84
Median 12	-	4,34	3,87
Average 22	0,4	4,19	3,52
Median 22	-	4,30	3,65
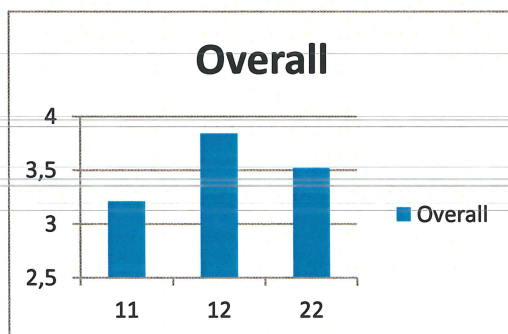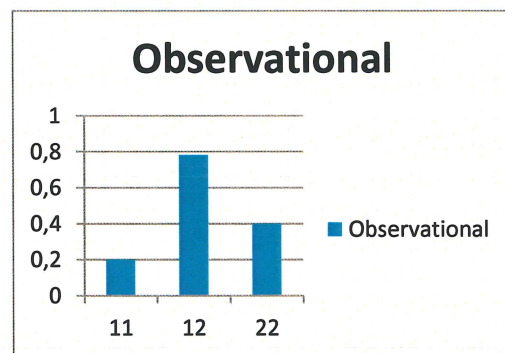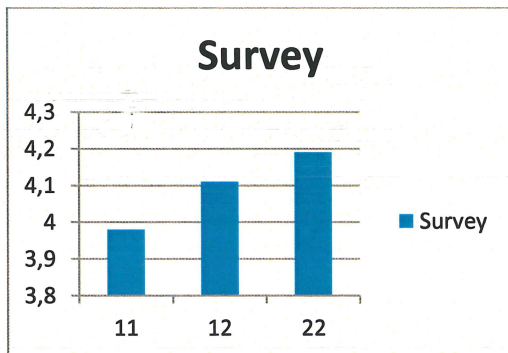
Figures 9-11: Average score for each genotype, using three different means of classification.

Association of DAT (exon 10, CFA34) with behavior

Three SNPs are located at positions 34:11235772, 34:11235810 and 34:11235835 in exon 10 of

the DAT-gene. The linkage phase of the SNPs are guanine at the first SNP, cytosine at the

second SNP and guanine at the third SNP for haplotype 1, and adenine at all three SNPs in

haplotype 2, all in complete linkage disequilibrium. The allele frequencies for haplotype 1 and

haplotype 2 were 0,67 and 0,33 respectively.

Table 13: DAT exon 10 haplotypes

	34:11235772	34:11235810	34:11235835
Haplotype 1 (H1)	G	C	G
Haplotype 2 (H2)	A	A	A

Because of the complete LD, haplotypes were studied in the association studies rather than single

SNPs. The association between the two haplotypes and phenotype was investigated, using the

same three phenotypic measures as previously described with the DRD2-gene.

Results DAT exon 10

Association between DAT-genotype and observed phenotype was detected. In the observational

scoring 32 Havanese were classified as anxious and 33 Havanese were classified as controls.

Dogs that are homozygote for haplotype 1 (11) had significantly larger risk of being anxious than

the heterozygotes (12) (OR: 3,2727, P-value: 0,0402), and heterozygotes (12) and homozygotes

(22) combined (OR: 3,1250, P-value: 0,0344). When comparing the two homozygotes (11 vs.

22), no significant association with phenotype was found (OR: 2,80, P-value: 0,1574), probably

because of the small number of 22-dogs (5 cases, 7 controls).

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Table 14: OR-tables DAT, observed phenotype

	Anxious	Controls		Anxious	Controls
H1H1	16	8	H1H1	16	8
H1H2	11	18	H1H2+H2H2	16	25

OR:3,27/P:0,0402   OR:3,13/P:0,0344

The average observational score for each genotype was calculated (Anxious=0, control=1).

Table 15: Average score for all genotypes (11, 12, 22)

Average 11 – observational score	0,35
Average 12 – observational score	0,63
Average 22 –observational score	0,58

No association between DAT-genotype and Survey score or Overall score was found.

Possible combined effect of DRD2 exon 2 and DAT exon 10
Even though the results from the DAT association study were somewhat inconclusive, the

possible combined effect of DRD2 and DAT on Observational classification was investigated.

Havanese that are homozygote for the risk haplotype in either DRD2 or DAT were compared to

Havanese that have at least one protective haplotype in each gene. Only 3 in 30 cases (10%) had

at least one protective allele in both DRD2 and DAT, as compared to 22 in 32 controls (69%).

The low number creates a problem when calculating the Odds Ratio, because preferably there

should be at least 5 dogs in each cell of the 2x2-table.

Table 16: OR-table DRD2 and DAT combined effect

	Cases	Controls
DRD$_2$11DAT11/ DRD$_2$11DAT12/ DRD$_2$11DAT22 DRD$_2$11DAT11/ DRD$_2$12DAT11/ DRD$_2$22DAT11	27	10
DRD$_2$12DAT12/DRD$_2$12DAT22/DRD$_2$22DAT12/DRD$_2$22DAT22	3	22

Odds ratio: 19,8 – P-value: <0,0001

This is however an indication that the DRD2-gene and the DAT-gene could have a greater effect

on phenotype when combined, that either of the genes have separately.

Polymorphism in intron 14 of DAT-gene
In addition we also sequenced a region in intron 14 (34:11243915) of the DAT-gene.

18 Havanese (7 cases and 11 controls) were sequenced using the primers from the article by Lit

et al. 2013 (20). All of them had the short variant without the poly(A) insertion or duplication.

Figure 12: variants found in the article by Lit et al. 2013 (20)Genetic variation in Havanese
A group of 21 Havanese was randomly selected as previously described. The heterozygosity for

each animal ranged from 0,5 to 0,89. The average degree of heterozygosity in the breed was

0,69. The average number of alleles per marker, detected in the breed, was 7,22 (range 3-12).

Coat variation in Havanese and the RSPO2 gene

The previously described insertion in the 3'UTR of the RSPO2-gene was investigated in 185

Havanese using the primers from the original article (5)

The short haired Havanese (n=3) in our material had a 142 basepair deletion. The 167 missing

basepairs that were described by Cadieu et al. (5), were replaced by a 25 basepair sequence in the

Havanese. In other words we found a 142 basepair deletion, and a 25 base pair substitution. The

25 base pair sequence was also found in the CanFam3.1 Boxer. The known "Short hair" carriers

(n=3) were heterozygote for the polymorphism as we expected, thus indicating that furnishings

(or "Long coat") is the dominant variant, similar to previous findings (5).

185 Havanese were tested for the RSPO2-polymorphisms. 3 short haired dogs, 47 carriers and

135 dogs that only carry the long variant, were detected in the material. 25,8 % of the long

coated individuals turned out to be carriers of the short coat variant. When including all 185

dogs, the allele frequencies are 0,857 and 0,143 for the long and short variant respectively.

Experimentally the frequency of the short allele was also calculated with the short haired dogs

excluded (as these may be overrepresented due to the fact that they were specifically recruited),

and this gave a frequency of 0,129.

The RSPO2-gene was completely associated with coat phenotype and estimated carrier status in

our material.

Two microsatellites were found in proximity to the RSPO2 polymorphism. Variation in these

microsatellites was detected. The Havanese differed from both the CanFam3,1 boxer, and among

themselves. No association between variants in these microsatellites and coat phenotype was detected.

Discussion

Significant differences in the level of anxiety were observed in individuals within the Havanese breed in this study. Nervousness is an undesired property and represents a welfare issue to both the dog and the owner. A relatively high frequency of mild to moderate anxiety has been recorded in the Havanese, with a significant variation between dogs. As with all genetic research on quantitative traits there are challenges with respect to classification.

DRD2

In the study on DRD2-variation we observed a significant association between phenotype and DRD2 exon 2 haplotypes, regardless of whether the classification was based on observation, survey score or overall score. The association is however a lot stronger when phenotype is classified based on the observational or overall score, compared to the survey score. This clearly illustrates how classification may influence results.

Observations made by the owner and observations made by an external evaluator have different strengths and weaknesses. The major weakness of owner evaluation may be that owners will evaluate dogs differently based on their skills and frame of reference. The evaluation my also not be completely objective.

Subjective owner evaluation (e.g. under-reporting of anxiety) could be a challenge if an owner/breeder were reporting several dogs from a certain line/genotype, which could lead to false association. The number of dogs per owner in this study was 2,08, and we therefore do not believe that the owner classification represents a systematic problem Repeatability is however suspected to be higher when only one person is doing the evaluation, as compared to each owner

evaluating their own dog. At the same time the owner knows the dog best, and has experienced the dogs' behaviour in various situations that are hard to recreate in a test situation.

In science there is often a need to systemise findings. Attempts are made to describe complex behavioural traits through standardised questionnaires or controlled tests. Because different aspects of behaviour could be influenced by different parts of the genome – studying clearly defined aspects of behaviour can be essential in detecting associated genes. There is however also indications that more subjective evaluations, preferably performed by only one or a few people, can detect behavioural patterns that the standardised test and questionnaires miss (34).

In our questionnaire, owners generally scored their dogs quite high (indicating low levels of anxiety). The average survey score was 4,07 and the median was 4,28. This could be due to a generally low level of anxiety in the studied dogs within the breed, because of the composition of the survey, or because owners have a tendency to choose the more positive alternative when considering two answers. It is most likely a combination of all three factors.

When considering only the survey based classification, as opposed to the observational or overall score, no association between anxiety and DRD2-genotype was found when the cut off was set close to the median.

This may be due to misclassification in the "middle 1/3" of the group. In other words; most people would agree on classification of a very mentally stable dog and also a severely anxious dog. There is probably a lower repeatability in scores from dogs that show weak signs of anxiety

compared to the extreme cases. In intermediate cases, the frame of reference of the person doing the evaluation will have a large impact on whether the dog is classified as a case or a control.

It appears that the survey based classification is accurate when comparing the "Bullet proof temperament"-dogs (dogs that don't show signs of anxiety in any situation) to "others". It may not be well suited for detecting the difference between normal dogs that show anxiety in some situations, and dogs that are a little bit more nervous than what is considered "normal", but still function quite well.

Because of the quantitative nature of anxiety traits, they are assumed to be influenced by many genes. The overall level of anxiety in an individual will therefore theoretically be determined by the frequency of protective or risk alleles at these loci (and of course environmental factors). Dogs at either end on the anxiety spectrum will with large probability have a higher number of risk alleles, or protective alleles. This will increase the likelihood of finding the suspected genotype at the gene of interest. This further explains how association might be stronger when the cut-off is set high.

The difference in results from the two different classification systems used in this study highlights some of the challenges in research on behaviour in dogs. Because of the sometimes small additive effects of markers, correct classification is crucial for successful detection of association.

DRD2-genotypes and anxiety
In the present material one aim was to compare the behaviour-phenotypes of the different DRD2-genotypes. Our studies indicated that homozygosity for haplotype 1 (11) is associated with

anxiety. The number of dogs homozygous for haplotype 2 (22) was too low to get secure estimates of odds ratio between the two homozygote groups. Most of the haplotype 2 alleles are included in the heterozygotes.

In genes with additive gene effects, with one risk allele and one protective allele, it is expected that the heterozygote has a phenotype that lies between the phenotype of the two homozygotes. On the other hand; heterozygotes are expected to be on the level of one of the homozygotes under dominance. Interestingly, in our material, there are indications that the heterozygotes are less anxious than either of the homozygotes groups.

To test this hypothesis, 12-dogs were compared to 22-dogs. If the mode of inheritance is complete dominance, one would expect an OR and P-value close to 1. If the mode of inheritance is partial dominance or no dominance, one would expect the OR to be less than 1 (when 22 is set as the risk genotype). To make sure there were enough (n=>5) 22-dogs in each cell of the 2x2-table the Overall Score Classification was used, as this was available for the largest number of dogs (n=157). The cut off was set at the median, also to make sure there were at least 5 22-dogs in both the case and control group.

Table 17: Genotypes and phenotype overall score classification

	Cases	Controls
$DRD_2$11	40	23
$DRD_2$22	13	8
$DRD_2$12	22	45

Table 18: OR-tables comparing 11 vs. 12-dogs and 22- vs. 12-dogs and association with phenotype. (Cut off set at median).

	Cases	Controls		Cases	Controls
$DRD_2$11	40	23	$DRD_2$22	13	8
$DRD_2$12	22	45	$DRD_2$12	22	45

OR:3,6/P:0,0006   OR:3,3/P:0,0207

The 12-dogs were, as illustrated in the table, not only significantly less anxious than 11-dogs (homozygote risk allele), but also significantly less anxious than the 22-dogs according to the Overall Score Classification.

When viewing the average and median of the different genotypes (11, 12, 22), using the three different classifications (Observational, Survey based and Overall score), there is no unambiguous indication of the mode of inheritance. The observational and overall score classification indicate that dogs with the 11-genotype are the most anxious, the 12-dogs are the least anxious, and the 22-dogs have an intermediate phenotype.

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For the Survey based classification the genotypes have a different behavior order: 11-dogs are the most anxious, the 12-dogs are intermediate and the 22-dogs are the least anxious.

The differences in distribution of genotype averages between various classifications could be due to the fact that the different scores could potentially measure different aspects of behavior, or with different associations to the tested genes. The different methods of classifications may also have different potential for misclassification.

The OR-values are generally higher, and the P-values are generally lower, when comparing 11-dogs with 12-dogs, than when comparing 11-dogs with 12- and 22-dogs combined, regardless of the classification. The only exception is when the 20% most anxious dogs were compared to "others". The fact that including the 22-dogs in the association study weakens the association when the cut of is set high, but strengthens the association when the cut off is set low, supports the theory that the 22-dogs have an intermediate phenotype.

In the Overall score classification an interesting observation was made; when the cut off was set at the median or higher, the frequency of 22-dogs was higher in the case group than the control group. When the cut off was set below the median, the frequency of 22-dogs was higher in the control group than in the case group. This also indicates that the 22-dogs have a phenotype that lies somewhere between the phenotype of the 11-dogs and the 12-dogs.

In the Observational and Survey based classification, the 22-indivduals appeared with similar frequencies in the case and control-groups, indicating an intermediate phenotype.
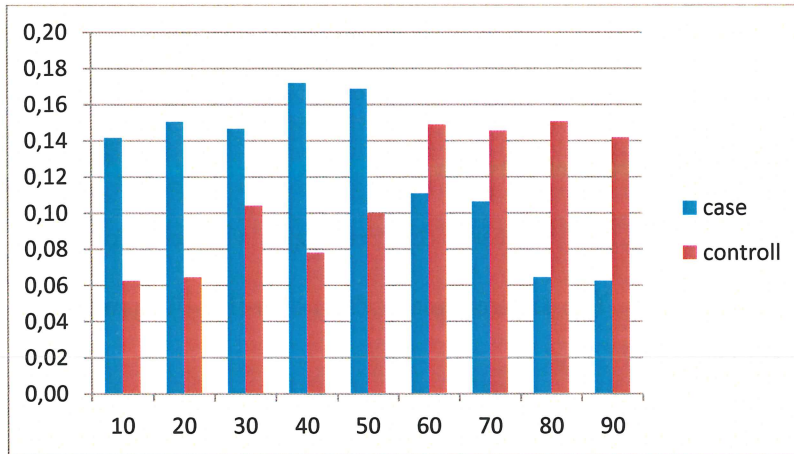
Figure 13: The frequency of 22-dogs in the case- and control-groups at various cut off points between top 10% and lower 10% in the overall score classification.

Interestingly, evidence of overdominance effects in DRD2 and other dopamine receptor genes have been found in several studies on humans and other species (6) (7).

One study showed that women that are heterozygote for polymorphisms in the DRD1-gene were significantly more attentive towards their infants than women from either homozygote group (21). Evidence of an overdominance effect in the DRD2-gene was also found in a study on cerebrospinal fluid levels of dopamine breakdown products. Heterozygotes had lower levels of the dopamine breakdown product homovanillic acid (HVA) than either homozygote group (13).

There are some indications of a similar distribution in our material.

To summarize; our findings clearly shows an association of DRD2 exon 2 to anxiety, and that being homozygote for haplotype 1 in exon 2 of the DRD2-gene significantly increases the risk of anxiety in the Havanese.

DAT

Significant association was detected between Observational score and DAT-genotype, whilst no association was detected for Survey score or Overall score.

We observed different odds ratios for the different phenotypic scores. This may be due to the fact that the different scores could potentially measure different aspects of behavior, or with different associations to the tested genes. There may also be differences in the potential misclassification in the Survey based classification compared to the Observational classification.

When comparing the odds ratios detected in the association studies on the DRD2-gene and DAT-gene, using the observational classification, OR was larger (OR: 14.2857, P-value: 0.0001) for the DRD2-gene than the DAT-gene (OR: 3,2727, P-value: 0,0402). A potentially larger degree of misclassification in the Survey score (compared to the observational score), could possibly be enough to disguise a small effect.

The 12- and 22-individuals both have higher average scores (indicating lower levels of anxiety) than the 11-individuals. The OR is slightly higher when comparing 11-dogs to 12-dogs, than when comparing 11-dogs to 12- and 22-dogs combined, but the difference is marginal (3,2727 vs. 3,1250).
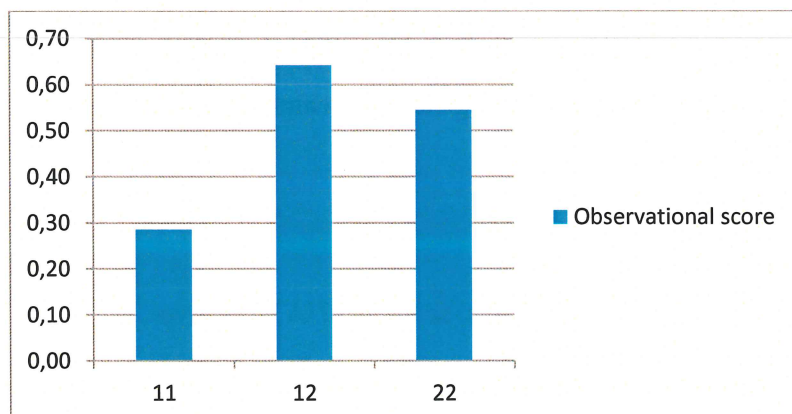
Figure 14: Average observational score all genotypes (11, 12, 22).

Our finding show that Havanese that are homozygote for haplotype 1 in exon 10 of the DAT-gene are significantly more likely to display anxious tendencies (observed phenotype) than Havanese that have at least one haplotype 2 allele.

The combined effect of DRD2 and DAT

Using the Observational classification, we studied the odds ratios of DRD2, DAT, and the two genes combined. The OR of DRD2 was between 14.2857 (P-value: 0,001) (11 vs. 12) and 10,8 (P-value: 0,0001) (11 vs. 12+22). The OR of DAT was between 3,2727 (P-value: 0,0402) (11 vs 12) and 3,1250 (P-value: 0,0344) (11 vs. 12+22). The OR of the two genes combined was 19.8 (P-value: <0,0001) (homozygote for risk allele in at least one gene vs. at least one protective allele in each gene). The results may be uncertain due to of a low number (n=3) of cases with protective alleles in the OR-table. There are however indications that the OR can be bigger when both the genes have favorable allele combinations.

Our results help to illustrate that genotyping of candidate genes involved in behavior may be valuable to predict risk of anxiety. In our group of cases (n=32) 10 dogs have at least one

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protective allele in DRD2 and 16 have at least one protective allele in DAT, but only 2 have protective alleles in both genes. In our group of controls (n=33) 5 dogs have only risk alleles in DRD2 and 8 dogs have only risk alleles in DAT, but only 3 have only risk alleles in both genes. To get a better estimate of the combined effect of both genes, a much bigger material would be needed. Genotyping several genes could give a rough estimate of a dog's risk of anxiety, and by including more genes there is potential to support traditional breeding with marker assisted selection.

Pedigree effects

We have considered possible genetic background-effects, and whether this could have influenced the results. The 188 Havanese come from over 60 different breeders. This indicates that even if some of them are related, most of them are not. Due to the fact that the breed has grown in popularity the last decade, breeders have frequently imported genetic material from abroad. At least 15% of the dogs are imported, and an even higher percentage is first generation after imported parents.

Due to frequent behaviour differences within a litter or a family, related dogs in the material are distributed among both the cases and the controls.

To investigate the possible "family effects" further, littermates with known phenotypic differences were investigated (7 litters, 24 dogs). Even within the litters, with a common genetic background, there was association between drd2- and DAT-genotype, and the level of anxiety the dogs were displaying. This supports a true effect of the markers, even when family effects are reduced.

There is a possibility that the frequency of anxious dogs is somewhat higher in this material than in the general population, because anxious dogs were specifically encouraged to participate. One could also imagine that owners of dogs that are anxious have more motivation to partake in studies on anxiety. On the other hand, owners and especially breeders might be reluctant to participate with an anxious dog to avoid a negative reputation. Most owners stated that their main motivation to participate was either to support research on the breed, or to get a free RSPO2-test. As previously described anxious individuals might be underrepresented in the samples that were collected at dog shows, because some owners feared that blood sampling of anxious dogs might negatively influence show performance.

However, even if there might be *some* deviation from the actual breed prevalence of nervousness in our material, this would probably not affect genotype-phenotype association, or the results of this study.

Conclusion

A relatively large number of Havanese was involved in the study. We believe our material is representative of the Norwegian Havanese population. Significant association between anxiety phenotypes and the DRD2- and DAT-gene were detected. Because the SNPs are synonymous the functional effect associated with the haplotypes are most likely due to linked mutations. Further research should be done to identify and study more polymorphisms associated with anxiety in Havanese. It would also be interesting to type different breeds of dogs, to investigate whether these genes, and even haplotypes, are associated with behavior in other breeds as well.

Genetic variation

The average degree of heterozygosity in the breed was 0,69. Our findings indicate that genetic variation in the Havanese breed is at a reasonable level. The possible genetic bottle neck that was suspected given the breed history does not yet appear to have had a detrimental effect on genetic variation in the breed. Possible explanations can be either that the bottle neck effect wasn't that significant to begin with or that fresh genetic material has been reintroduced (through later imports from Cuba or from other breeds) in later years.

RSPO2

Based on our findings, combined with previous findings (5), there is reason to believe that the RSPO2 polymorphisms truly are responsible for coat variation in Havanese. Even though the number of short haired Havanese (n=3) and known carriers (n=3) in our material is relatively low there is a complete association between RSPO2 genotype and phenotype/estimated carrier status in our study.

The estimated allele frequencies of the RSPO2 polymorphism are 0,857 and 0,143 for the long and short variant respectively. This frequency of the short allele could be an overestimate as two of the three short haired individuals were recruited specifically for their short coat, because their DNA was needed for the development of the DNA-test. Even if all of the short haired dogs were excluded from the study though, the frequency of the short allele is still 0,129.

The rest of the dogs (n=182) were recruited randomly from the population for the anxiety project. Because some of the samples were collected at dog shows, and short haired Havanese don't participate in dog shows, short haired dogs could be underrepresented. However, because

short haired Havanese occurs very seldom, some selection error of the short haired individuals should not impact the allele frequency to a great extent. The ratio of long haired carriers and long haired non-carriers will have the greatest impact in allele frequency, and we believe this ratio is representative in our study.

With this allele frequency we would, according to the Hardy-Weinberg Equilibrium, expect approximately 8 short haired Havanese puppies to be born in Norway each year (assuming around 400 registrations per year). This is in accordance with the estimated prevalence.

The Swedish Havanese and Bolognese Club (BBHC) has previously written an article about short coats in the Havanese (36). In the article BBHC discuss the possible mode of inheritance of this trait. They suggest that the trait is not autosomal recessive because they know of only 35 short haired Havanese in a 13 year period with 6500 registrations (0,5%). This frequency of short haired dogs does however fit nicely with the Hardy-Weinberg equilibrium in a situation with 13% carriers.

It is also very unlikely that the Breed Club know of all cases of an "undesired trait" like this, because some breeders would not want to share that kind of information with the breed club. If BBHC know of 35 cases, one would expect the real number of cases to be higher. If the true prevalence of short haired Havanese is larger than 0,5%, the estimated percentage of carriers would also be higher than 13%. In our study, we found that 25,8 (47/135+47) of long haired Havanese are carriers of the short variant.

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Our results therefore indicate that the RSPO2 is responsible for the short coat in Havanese, and that the mode of inheritance is in accordance with complete dominance with the long coat being the dominant allele.

Acknowledgments

Anxiety in Havanese

Sammendrag

Angst og nervøsitet forekommer hyppig hos hunder, og representerer viktige velferdsproblemer

for både hund og eier. Mild til moderat angst forekommer relativt hyppig hos Bichon Havanais,

til tross for at en sosial og utadvendt personlighet er regnet som et viktig kjennetegn ved rasen.

Forskning har vist signifikant assosiasjon mellom atferd og genotype i dopaminreseptor 2-genet

(DRD2) og dopamintransportergenet (DAT) hos ulike arter. I dette studiet har vi sett på variasjon

i disse genene hos Bichon Havanais, og potensiell assosiasjon med engstelig fenotype. I dette

studiet fant vi at:

- Det er stor grad av variasjon i dopaminreseptor 2-genet, DRD2, og

  dopamintransportergenet, DAT, hos Bichon Havanais.

- Det er signifikant assosiasjon mellom haplotyper i exon 2 i DRD2-genet og nivået av

  angst hos ulike individer innen rasen, uttrykt både i form av spørreskjema besvart av eier,

  og observert fenotype.

- Det er signifikant assosiasjon mellom haplotyper i exon 10 i DAT-genet og observert

  nivå av angst hos Bichon Havanais.

I tillegg studerte vi en mutasjon assosiert med kort pels, og vi vurderte genetisk variasjon i rasen.

Resultatene viste at en mutasjon i RSPO2-genet co-segregerte med kort pels hos Bichon

Havanais, og at frekvensen av denne mutasjonen var 0,143 i den studerte populasjonen. Genetisk

variasjon ble målt som gjennomsnittlig grad av heterozygoti (0,69), og det var ingen indikasjon

på lav genetisk variasjon i rasen.

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Supporting material

Phenotype scores and genotype of all Havanese (n=188)

Id	Questionnaire	Observed	Overall	DRD2 EX2	DAT EX10
1	4,78		3,89	H1H2	H1H1
2	4,89	1	4,94	H1H2	H1H2
3	4,57		3,79	H1H1	H1H1
4	4,95		3,98	H1H2	H1H2
5	3,70		3,35	H1H2	H1H1
6	3,04	0	2,02	H1H1	H1H2
7	4,27		3,63	H2H2	H1H2
8	4,78	1	4,89	H1H1	H1H2
9	4,44	0	2,72	Other	H1H1
10	4,78	0	3,89	H2H2	H1H1
11	4,88		3,94	H1H2	H1H2
12	4,85		3,93	H1H2	H1H2
13	4,92		3,96	H2H2	H1H1
14	4,15	1	4,57	H1H2	H1H1
15	4,96	1	4,98	H2H2	H1H1
16	3,52	0	2,26	H1H2	H2H2
17	4,30		3,65	H2H2	
18	4,91		3,96	H1H2	H1H1
19	3,36		3,18	H1H2	H1H2
20	4,26	1	4,63	H1H2	H1H2
21	3,85		3,43	H1H1	H1H1
22	4,74		3,87	H1H2	H1H1
23	2,64	0	1,82	H1H1	H1H1
24	3,04	0	2,02	H1H1	H1H2
25	4,23	1	4,62	H1H1	H1H2
26	4,35	1	4,67	H1H2	H1H1
27	3,81	1	4,41	H1H2	H1H2
28	3,74		3,37	H1H1	H1H1
29	2,12		2,56	H1H1	H1H2
30	4,74	1	4,87	H1H2	H1H2
31	2,96	0	1,98	H1H2	H2H2
32	4,74	1	4,87	H1H2	H1H2
33	4,19		3,59	H1H1	H1H2
34				H1H1	H1H2

35				H2H2	
36				Other	
37				H1H2	H1H1
38				H1H1	H1H2
39				H2H2	
40					
41					
42		0	1,00	H1H1	H1H2
43	4,74	0	2,87	H1H1	H2H2
44	3,88	0	2,44	H1H1	H2H2
45				H1H2	H1H2
46	4,08	1	4,54	H1H2	H2H2
47	4,00		3,50	H1H1	H1H2
48	4,31	1	4,66	H1H2	H1H2
49	3,70	1	4,35	H1H2	H1H2
50	3,88	1	4,44	H1H2	H1H2
51	4,26	1	4,63	H1H2	H1H2
52	3,15	0	2,07	H1H1	H1H1
53				H1H1	H1H1
54	4,54		3,77	H2H2	H2H2
55	4,85		3,93	H2H2	H1H2
56	3,96		3,48	H2H2	H1H2
57	4,42		3,71	H1H1	H1H1
58	4,31		3,65	H2H2	H1H1
59	4,43		3,71	H1H1	H1H2
60	4,30		3,65	H2H2	H1H2
61		1	5,00	H1H2	H1H1
62	4,67		3,83	H1H2	H1H2
63	4,31	0	2,65	H1H1	H1H1
64				H1H1	H1H1
65	4,59		3,80	H1H2	H1H1
66	4,52		3,76	H1H2	H1H1
67	3,54		3,27	H1H1	H1H1
68		1	5,00	H1H1	H1H1
69				H1H2	H1H1
70				H2H2	H1H2
71	4,41	1	4,70	H2H2	H1H2
72	3,11	0	2,06	H1H1	H1H1
73	3,35	0	2,17	H1H1	H2H2
74	4,56	1	4,78	H1H2	H2H2
75	4,56		3,78	H1H1	H1H1

76	4,31		3,65	H1H2	H1H1
77	3,81		3,41	H1H1	H1H1
78	3,35		3,17	H1H1	H1H1
79	4,85	1	4,93	H1H2	H1H2
80	3,04		3,02	H2H2	H1H2
81	4,81	1	4,91	H1H1	H1H1
82				H1H1	H1H1
83	3,56	0	2,28	H1H1	H1H1
84	2,96		2,98	H1H2	H1H2
85				H1H2	H1H1
86	4,92		3,96	Other	H1H2
87	4,41	0	2,70	H1H2	H1H1
88	4,30	0	2,65	H1H1	H1H2
89	3,15	0	2,07	H1H2	H1H2
90	4,04		3,52	H1H1	H1H1
91		0	1,00	H1H1	H1H1
92	3,35	0	2,17	H1H1	H1H2
93	4,41	1	4,70	H1H2	H2H2
94	2,33	0	1,67	Other	H1H2
95				H1H1	H1H1
96	3,64	0	2,32	H1H1	H1H2
97	4,68	1	4,84	H1H2	H2H2
98	3,19		3,10	H1H2	H1H1
99	4,78		3,89	H1H1	H1H2
100	4,63	1	4,81	H1H1	H1H1
101	4,59		3,80	H1H1	H1H2
102	4,44		3,72	H1H1	H1H2
103	3,59		3,30	H1H1	H1H2
104	3,52		3,26	H1H2	
105	4,27		3,63	H1H1	H1H2
106	4,70		3,85	H1H2	H1H1
107	4,59		3,80	H1H2	H1H2
108	4,56		3,78	H1H2	H1H1
109	4,41		3,70	H1H2	H1H2
110	4,22	0	2,61	H1H1	H1H2
111	4,52	0	2,76	H1H1	H1H1
112	4,00	0	2,50	H1H1	H1H2
113				H1H2	H1H2
114	4,46		3,73	Other	H1H2
115	3,89	1	4,44	Other	H1H2
116	4,48		3,74	H2H2	H1H1

117	2,77		2,88	H1H1	H1H2
118	3,65		3,33	H1H1	H1H2
119	4,15		3,57	H2H2	H1H2
120					
121	4,33		3,67	H2H2	H2H2
122	3,19		3,10	H1H2	H1H2
123				H1H2	H1H1
124				H1H1	H1H2
125				H1H1	H2H2
126	4,44		3,72	H1H1	H1H2
127	3,44		3,22	H2H2	H1H2
128	4,59	1	4,80	H1H2	H1H2
129	4,00	0	2,50	H2H2	H1H1
130	2,73		2,87	H1H2	H1H2
131				H1H1	H1H1
132	3,69		3,35	H1H2	H2H2
133	3,19		3,09	H1H2	H1H1
134	4,78		3,89	H1H2	H1H2
135	1,85	0	1,43	H1H2	H1H1
136	4,38	1	4,69	H1H2	H2H2
137	3,58		3,29	H1H1	H1H1
138	3,41		3,20	H1H2	H1H2
139	4,54		3,77	H1H1	H1H1
140	4,73		3,87	H1H1	H1H1
141	4,36		3,68	H1H2	H1H1
142	4,35		3,67	H1H1	H1H2
143				H1H2	H1H2
144	3,74		3,37	H1H1	H1H2
145	4,58		3,79	H1H1	H1H2
146				H1H2	H1H1
147				H1H1	H1H1
148	3,27		3,13	H1H1	H1H1
149	4,11		3,56	H1H1	H1H1
150	4,77	1	4,88	H1H2	H2H2
151	4,33		3,67	H1H2	H1H1
152	2,46		2,73	H1H2	H1H2
153	4,27		3,63	H1H2	H1H1
154	3,88		3,44	H1H2	H1H2
155	4,70		3,85	H1H2	H1H1
156	3,58		3,29	H2H2	H1H1
157	3,89		3,44	H1H2	H1H1

83

158		1	5,00	H1H2	H1H2
159		1	5,00	H1H2	H1H2
160	4,84		3,92	H1H2	H1H1
161	3,46		3,23	H2H2	H1H2
162	5,00		4,00	H1H2	H1H1
163	5,00		4,00	Other	H1H1
164	4,85		3,93	H1H1	H1H2
165	4,23		3,62	H1H1	H1H1
166	3,28	0	2,14	H1H2	H1H1
167		0	1,00	H2H2	H1H1
168	4,67		3,83	H1H1	H1H1
169	4,58		3,79	H1H1	H1H1
170	4,67		3,83	H1H1	H1H1
171				H1H2	H1H1
172	4,35		3,67	H1H1	H2H2
173	4,42	1	4,71	H1H2	H1H1
174	3,38	0	2,19	H1H1	H1H1
175	4,19	1	4,60	H1H2	H2H2
176	1,93	0	1,46	H1H2	H1H1
177					
178					
179					
180					
181					
182	3,54		3,27	H1H1	H1H2
183	3,85	0	2,42	H1H1	H1H2
184	3,65		3,33	H2H2	H1H2
185	4,44		3,72	H1H1	H1H2
186	4,56		3,78	H1H2	H1H1
187	3,13		3,06	H1H1	H1H2
188	4,22	1	4,61	H1H2	H1H2

Anxiety in Havanese

Owner questionnaire:

Atferd hos Havanais

Tusen takk for at du har gitt blodprøve/svaber fra din hund til prosjektet "Genetiske faktorer som påvirker angst hos Bichon Havanais". For å kunne bruke blodprøven I forskning trenger vi å kunne knytte informasjon om atferd til hver hund. Vedlagt følger derfor noen spørsmål om din hunds atferd (og helse) som er viktige å ha svar på for at vi skal ha muligheten til å undersøke sammenhenger mellom egenskapen og gener fra blodprøvene. Dersom du har gitt prøver fra flere hunder må du fylle ut et eget skjema for hver hund.

Alle opplysninger om hund og eier behandles konfidensielt, og er bare tilgjengelige for dem som arbeider med prosjektet på Veterinærhøgskolen. Ingen informasjoner om enkelthunder, eier, oppdrett vil bli tilgjengelige for andre.

2) Hundens kallenavn (navn i dagligdags bruk)

3) Registreringsnummer eller stamtavlenavn

4) Eiers navn?

5) Eiers e-postadresse?

6) Eiers telefonnummer?

Anxiety in Havanese

7) Hundens fødselsår

Velg alternativ [⌄]

8) Fødselsdato (bruk formatet dd.mm.yyyy, altså som f.eks 24.12.2012, dvs 8 tall og to punktum)

9) Hundens kjønn?

○ Tispe   ○ Hannhund

10) Er din hund kastrert / sterilisert?

○ Ja   ○ Nei

11) Når overtok du hunden?

○ Eget oppdrett - hatt hunden siden fødsel

○ Overtok ved ca. 8 uker

○ Overtok ved ca. 10 uker

○ Overtok ved ca. 12 uker

○ Overtok ved 3 - 6 måneder

○ Overtok da hunden var 6-12 måneder

○ Overtok da hunden var over et år

○ Vet ikke

12) Har hunden noen gang deltatt på et valpekurs eller dressurkurs?

○ Ja   ○ Nei   ○ Vet ikke

13) Har hunden vokst opp med/levd sammen med flere hunder i husholdningen?

○ Nei

○ Ja, en hund til i huset

○ Ja, flere hunder

14) Hvem har hunden vokst opp med/levd sammen med (flere kryss mulig)?

☐ Barn under 10 år

☐ Barn over 10 år

☐ Voksne kvinner

☐ Voksne menn

15) Har hunden noen gang blitt (alvorlig) angrepet av en annen hund (flere kryss mulig)?

☐ Nei

☐ Ja, før den fylte 1 år

☐ Ja, etter at den fylte 1 år

☐ Ja, Flere ganger

☐ Vet ikke

16) Har hunden noen gang blitt skremt eller skadet av et barn (flere kryss mulig)?

☐ Nei

☐ Ja, før den fylte 1 år

☐ Ja, etter at den fylte 1 år

☐ Ja, Flere ganger

☐ Vet ikke

17) Har hunden noen ganger blitt skremt eller skadet av en voksen (flere kryss mulig)?

☐ Nei

☐ Ja, før den fylte 1 år

☐ Ja, etter at den fylte 1 år

☐ Ja, Flere ganger

☐ Vet ikke

19) Hvordan er du fornøyd med hundens generelle atferd ?

○ Meget godt   ○ Godt   ○ Middels   ○ Dårlig   ○ Svært dårlig

20) Hunden min er

	Ikke riktig	Lite riktig	Hverken riktig eller galt	Ganske riktig	Meget riktig
Redd for fremmede hunder	○	○	○	○	○
Redd for fremmede barn	○	○	○	○	○
Redd for fremmede kvinner	○	○	○	○	○
Redd for fremmede menn	○	○	○	○	○
Redd for mennesker med uvanlig oppførsel/utseende	○	○	○	○	○
Engstelig på støyende/stressende steder	○	○	○	○	○
Glad i barn	○	○	○	○	○

21) På tur med hunden vil den ofte

	Ikke riktig	Lite riktig	Hverken riktig eller galt	Ganske riktig	Meget riktig
Trekke seg unna når fremmede hunder vil hilse	○	○	○	○	○
Bjeffe mot andre hunder	○	○	○	○	○
Trekke seg unna når fremmede mennesker vil hilse	○	○	○	○	○
Bjeffe mot andre mennesker	○	○	○	○	○

22) Hunden min kommer godt overens med fremmede hunder

○ Ikke riktig   ○ Lite riktig   ○ Hverken riktig el galt   ○ Ganske riktig   ○ Meget riktig

23) Hunden min bjeffer mye

○ Ikke riktig   ○ Lite riktig   ○ Hverken riktig el galt   ○ Ganske riktig   ○ Meget riktig

24) På valpekurs/dressurkurs var hunden redd for andre hunder

○ Ikke riktig

○ Lite riktig

○ Hverken riktig el galt

○ Ganske riktig

○ Meget riktig

○ Vet ikke

Anxiety in Havanese

25) Viser hunden angst i noen av situasjonene under?

	Nei	Mild	Moderat	Sterk	Meget sterk	Vet ikke
Sterk trafikk	○	○	○	○	○	○
Tordenvær	○	○	○	○	○	○
Skudd/høye smell	○	○	○	○	○	○
Fyrverkeri	○	○	○	○	○	○

26) Hunden bruker lang tid på roe seg/slappe av hvis den blir skremt

○ Ikke riktig
○ Lite riktig
○ Hverken riktig eller galt
○ Ganske riktig
○ Meget riktig

27) Hunden er redd for å være alene hjemme/har separasjonsangst

○ Ikke riktig
○ Lite riktig
○ Hverken riktig eller galt
○ Ganske riktig
○ Meget riktig

28) Hunden min kan beskrives som

	Ikke riktig	Lite riktig	Hverken riktig eller galt	Ganske riktig	Meget riktig
Modig	○	○	○	○	○
Modigere enn andre Havanais	○	○	○	○	○
Forsiktig	○	○	○	○	○
Skvetten	○	○	○	○	○
Engstelig i enkelte situasjoner	○	○	○	○	○
Pysete/pinglete	○	○	○	○	○

29) På utstilling sliter jeg med å "få opp halen"

○ Ikke riktig
○ Lite riktig
○ Hverken riktig el galt
○ Ganske riktig
○ Meget riktig
○ Vet ikke, deltar ikke på utstilling

30) Hunden min har et høyt aktivitetsnivå

○ Ikke riktig   ○ Lite riktig   ○ Hverken riktig el galt   ○ Ganske riktig   ○ Meget riktig

31) Det var (nesten) alt. Kan du tenke deg å svare på to helsespørsmål før du sender inn?

○ Ja   ○ Nei

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32) Hvordan er hundens generelle helse?

○ Meget god   ○ God   ○ Middels   ○ Dårlig   ○ Meget dårlig

33) Kryss av hvis din hund har hatt langvarige/gjenntakende eller alvorlige problemer med noen av det følgende (flere valg mulig)

☐ Hud-hårlag-ører

☐ Fordøyelsesproblemer

☐ Luftveier-nese-lunger

☐ Øyesykdommer/øyekatarr

☐ Ledd/skjelett/muskel

☐ Nervesystemet

☐ Stoffskifte-hormoner

☐ Urinveier-nyre

☐ Reproduksjon/forplantning/testikler/livmor/jur

☐ Hudkuler/svulster

☐ Andre svulster/kreft

☐ Annet

34) Takk for hjelpen! Ved å trykke på "send" godtas at blodprøven brukes til forskning (men ingen informasjon om enkelthunder blir gitt/presentert noe sted).