Genetic studies of canine anxiety

Philosophiae Doctor (PhD) Thesis

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Oslo, January 2016

Linn Mari Storengen

SUMMARY

Canine anxiety is a common behavioral problem in dogs, affecting the dog's welfare and health, and causing a strain in the relationship between the dog and its owner. Undesirable behaviors, including anxiety disorders, is thus an important cause of euthanasia and relinquishment. Common anxiety disorders include noise sensitivity, separation anxiety, generalized anxiety disorder and phobias. A higher prevalence of canine anxiety is observed within certain dog breeds, and an underlying genetic predisposition is very likely. Specific genetic risk factors however, largely remain to be identified. The present thesis addresses canine behavioral genetics, with a special focus on canine anxiety.

In paper I, a descriptive study of dogs diagnosed with separation anxiety showed that some breeds had a higher prevalence of separation anxiety. Over half of the dogs were male and more male dogs diagnosed with separation anxiety were neutered compared to female dogs in the material. The majority of the dogs had other behavioral problems in addition to separation anxiety, the most common co-morbid diagnosis was noise sensitivities.

The prevalence of noise sensitivities was studied in seventeen breeds including over 5,000 dogs in paper II. The major focus was on noise from fireworks, loud noises such as bangs/gunshots, thunderstorms and heavy traffic. In general, the frequency of fearful dogs was high, on average approximately 23% were reported to be fearful of noises, with fireworks being the category where most dogs were reported to be fearful. Significant differences in frequencies of fearful dogs were also found between the breeds. Fearfulness in the different categories of noise co-occurred and there was a significant trend of increasing fear with age. In this study female dogs had higher odds of being fearful compared to male dogs, and neutered dogs were generally more sensitive to noise than intact dogs. The dogs most fearful of noises also had higher odds of showing separation related behavior.

In paper III a genome-wide association analysis was performed in five breeds to identify (possible) genomic regions associated with canine anxiety. The study

showed that allele frequencies varied greatly, both within breed and between the breeds, as well as between cases and controls. However, the results did not show robust associations between the phenotype and genetic markers. The study indicates that the genetic heterogeneity between the breeds will make it difficult to achieve genome-wide significance when including several unrelated breeds, and thus an across breed analysis will be suboptimal to detect associations. In situations with limited sample sizes from the same breed, candidate gene studies would probably be a better approach to detect associations to behavior traits.

In the final paper a candidate gene approach was used to study genetic risk factors associated with canine anxiety in five breeds and there was found an association with generalized anxiety and noise sensitivity to the dopamine receptor gene *DRD2* in three breeds. The dopamine receptor is important in the regulation of dopamine levels in the synapses in the brain, and human studies have found association between dopamine receptor density and anxiety disorders.

The present work has provided important findings in canine behavioral research, including significant breed differences in the prevalence of canine anxiety, as well as demonstrated highly different allele frequencies between and within breeds, and between cases and controls with regards to noise sensitivity. In addition, association between SNPs and canine anxiety was found in the dopamine receptor gene *DRD2* in several breeds. Finding genetic alterations underlying behavioral problems has the potential to contribute to enhancements in diagnosis, and in a long-term perspective improving the health and welfare of dogs.

SUMMARY IN NORWEGIAN (SAMMENDRAG)

Angstlidelser hos hund er veldig vanlig og påvirker både hundens helse og velferd, og kan gi ulike typer avvikende atferd. Problematferd kan føre til en negativ innflytelse på forholdet mellom hund og eier, og er en viktig årsak til at hunder avlives. Angstlidelser på hund inkluderer separasjonsangst, frykt for høye lyder, generalisert angstlidelse og fobier. Noen hunderaser har høyere forekomst av angstlidelser, og det er sannsynlig at en genetisk predisposisjon er underliggende. Spesifikke genetiske risikofaktorer gjenstår fremdeles å bli identifisert. Dette doktorgradsarbeidet omhandler atferdsgenetikk på hund, med et spesielt fokus på angstlidelser.

Den første studien i denne avhandlingen er en deskriptiv studie av hunder diagnostisert med separasjonsangst og viser at noen raser har en høyere forekomst av separasjonsangst. Studien viste at hannhunder var overrepresentert i materialet, i tillegg var flere hannhunder som ble diagnostisert med separasjonsangst kastrerte sammenlignet med tisper i materialet. Majoriteten av hundene hadde andre atferdsdiagnoser i tillegg til separasjonsangst, den vanligste diagnosen var lydsensitivitet.

Prevalensen av lydsensitivitet ble videre studert i den andre delstudien der 17 hunderaser med over 5000 hunder totalt var inkludert. Hovedfokus var på fire kategorier av lyder; nyttårsraketter, høye lyder og skudd, tordenvær og sterk trafikk. Frekvensen av engstelige hunder var rundt 23%, og flest hunder viste angst for nyttårsraketter. Signifikante forskjeller i forekomsten av engstelige hunder ble funnet mellom rasene. Det ble vist en positiv korrelasjon mellom angst for lyd i de ulike kategoriene, det vil si hvis hunden var engstelig for en type lyd økte sannsynligheten for at den også var engstelig for andre typer lyder. Det ble også funnet en trend med økende frykt korrelert med alder. Tisper hadde en høyre risiko for å være engstelige sammenlignet med hannhunder, og kastrerte hunder var generelt mer sensitive for lyd enn ukastrerte hunder. Hundene som var klassifisert som engstelige i denne studien hadde også en høyere risiko for å vise separasjonsrelatert atferd.

I den tredje delstudien ble fem ulike raser med over 400 hunder totalt inkludert i en GWAS for å identifisere regioner i genomet som kunne vært assosiert med angstlidelsen lydsensitivitet. Resultatene viste ingen robuste assosiasjoner mellom atferdsegenskapen og genetiske markører. Det ble funnet at allelfrekvensene varierte betraktelig mellom rasene. Innenfor enkeltrasene kunne det også være store variasjoner i allelfrekvenser mellom de hundene som viste frykt for høye lyder og de hundene som ikke var engstelige i det hele tatt. Denne genetiske heterogeniteten mellom raser vil trolig gjøre det vanskeligere å kunne finne statistiske signifikante koblinger mellom atferd og markører i studier der flere hunderaser er inkludert, selv om totalantallet av hunder inkludert er høyt. Studien bekreftet at kandidatgenstudier kan være en bedre tilnærming for å finne koblinger mellom genetiske markører og atferdsegenskaper dersom man ikke får tak i et tilfredsstillende materiale med mange nok hunder innenfor samme rase.

I den siste studien ble det derfor gjennomført en studie av nedarvede mutasjoner i gener kjent for å være assosiert med angst. Flere av rasene som var inkludert viste assosiasjon mellom generell engstelighet og lydsensitivitet og genet for en dopaminreseptor (*DRD2*). Dopaminreseptorer er viktige i reguleringen av dopaminnivåer i synapsene som finnes i hjernen, humanstudier har blant annet funnet koblinger mellom tetthet av dopaminreseptorer og angstlidelser.

Resultatene fra dette doktorgradsarbeidet har bidratt med viktige funn innen atferdsforskning på hund, det ble funnet signifikante forskjeller i forekomsten av frykt for høye lyder mellom ulike raser, i tillegg viste den ene studien av lydsensitivitet store forskjeller i allelfrekvenser mellom og innad i rasene inkludert, og også mellom kasus og kontroll. Det ble også funnet assosiasjon mellom SNPer i dopaminreseptorgenet *DRD2* og angst hos flere raser. Avdekking av genetiske risikofaktorer for atferdsproblemer vil øke forståelsen av disse sykdommenes etiologi og vil være et viktig grunnlag for utvikling av bedre diagnostikk og risikoestimater, noe som på sikt kan bidra til redusert forekomst av angst og bedring i hunders helse og velferd.

ABBREVIATIONS AND GENETIC TERMS

C-BARQ	Canine behavioral assessment and research questionnaire
CFA	Canine chromosome
CNV	Copy number variation
DRD2	Dopamine receptor D2
GWAS	Genome-wide association study
ISWT	Irish softcoated wheaten terrier
LD	Linkage disequilibrium
MAF	Minor allele frequency
MDS	Multi dimensional scaling
MicroRNA	Non-coding RNA playing a key role in regulation of gene expression
mRNA	Messenger RNA
NB	Norwegian Buhund
NGS	Next-generation sequencing
NKK/NKC	The Norwegian Kennel Club
NSDTR	Nova Scotia duck tolling retriever
SA	Separation anxiety
SB	Staffordshire bull terrier
SNP	Single nucleotide polymorphism
QQ plot	Quantile quantile plot

LIST OF PAPERS

Paper I

A descriptive study of 215 dogs diagnosed with separation anxiety
Linn Mari Storengen, Silje Christine Kallestad Boge, Solveig Johanne Strøm, Gry Løberg, Frode Lingaas
Appl Anim Behav Sci. 2014 vol. 159 pp. 82-89

Paper II

Noise sensitivity in 17 dog breeds: Prevalence, breed risk and correlation with fear in other situations
Linn Mari Storengen, Frode Lingaas
Appl Anim Behav Sci. 2015 vol. 171 pp. 152-160

Paper III

A genome-wide association study for noise sensitivity in 5 dog breeds
Linn Mari Storengen, Elin Kristiansen, Ernst Otto Ropstad, Frode Lingaas
Manuscript

Paper IV

***DRD2* is associated with anxiety in some dog breeds**
Kim Bellamy, Linn Mari Storengen, Karin H. Westereng, Ellen Arnet, Frode Lingaas
Manuscript

INTRODUCTION

CANINE GENETICS

Domestication of the dog

The domestic dog (*Canis familiaris)* belongs to the genus *Canis* which includes wolves, coyotes and jackals in addition to the dog. The process of dog domestication is still not fully understood and many aspects remain elusive, but it was probably a result of a mutually beneficial relationship with humans, sharing living space and food sources. Molecular genetic research suggests that dogs originated from the grey wolf (*Canis lupus)* (1,2). There is still no consensus on the exact time of domestication, time estimates from different studies range from 11,000 to more than 100,000 years ago (2-4), but there is agreement among archeologists and geneticists that dogs evolved from Eurasian grey wolves at least 15,000 years ago (5). Strong evidence points to Central Asia as the geographic origin of domestication (6). Since the first domestication, humans have selectively bred dogs that excel at herding, hunting and obedience, and along this process the creation of the dog breeds we see today have arisen (1) (Figure 1).

Population bottlenecks

The modern dog consists of over 400 breeds, each with specific behavioral and physiological attributes (1,7). It is the most diverse domestic species, with an impressive span in breed size and conformation, and the different phenotypes/breeds show various degrees of genetic relatedness (8)(Figure 1). Two population bottlenecks in the dog population shaped the haplotype structure in modern dog breeds (Figure 2). A population bottleneck is characterized by a marked reduction in population size followed by an expansion originating from a small number of random breeders from the original population. When this occurs longer linkage disequilibrium (LD – the non-random association between two or more loci) patterns are created because the population is left with fewer haplotypes (9). The first occurred at the initial domestication of dogs from the wolf, when a few domesticated wolves became the founders of a larger population of dogs, this population remained for a period of thousands of years, and during this time dogs were randomly mated with other dogs and also occasionally with wild wolves (Figure 2). The second bottleneck found place when the breeds were created (Figure 2). The modern dog breeds have developed over the past few hundred years (10). Many of the breeds derived from a small number of founders that best represented the physical or behavioral traits breeders wished to feature in a given breed, such as the ability to hunt, herd, guide and guard (11).

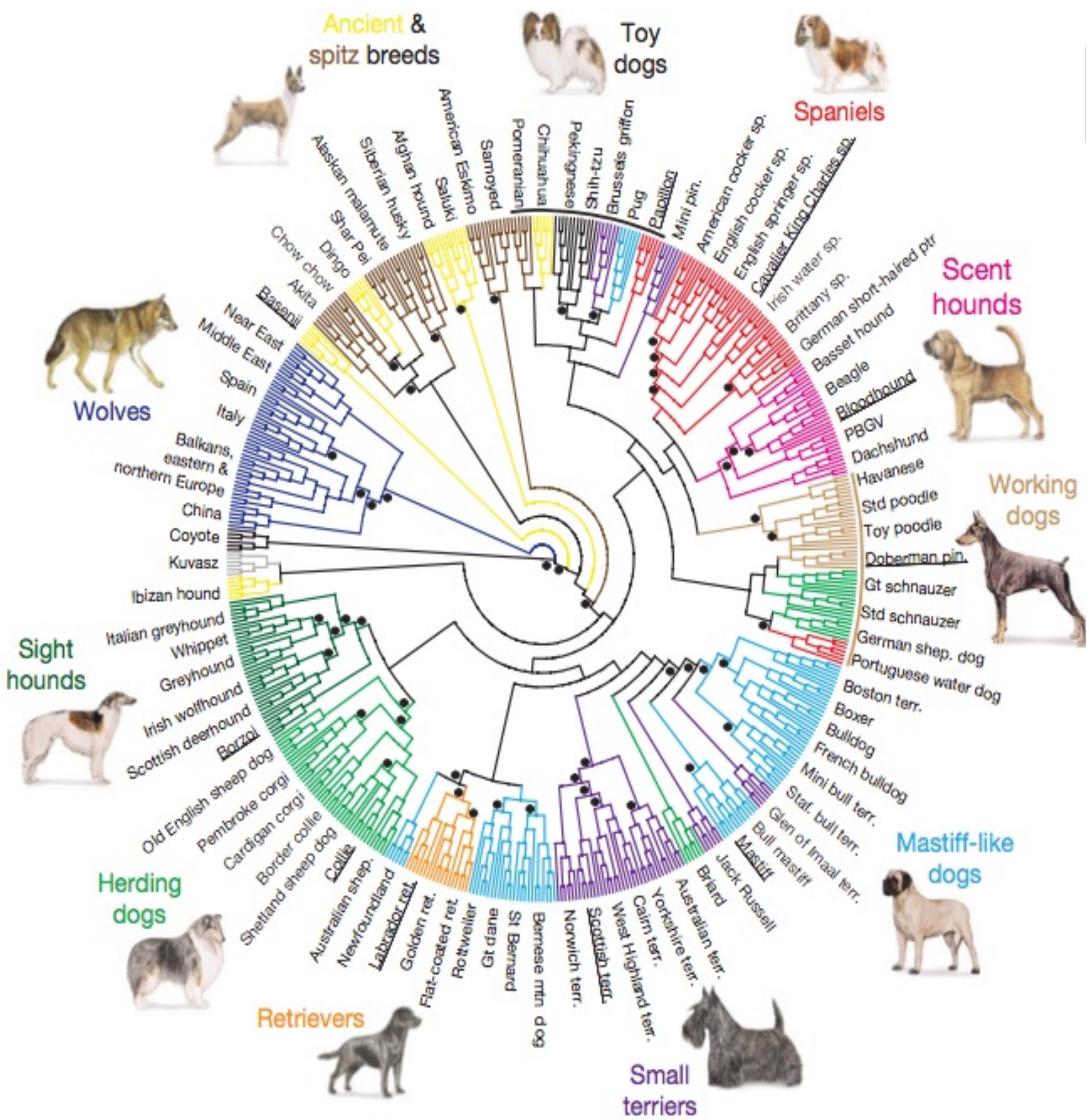

Figure 1 Haplotype-sharing cladogram of domestic dogs and grey wolves

Neighbour-joining trees of domestic dogs and grey wolves (n = 6 for each breed and wolf population) showing a haplotype-sharing cladogram. Breeds that probably share common founders are placed in the same color groupings. Figure modified from vonHoldt et al (8). Reused with permission from Science.

In the early 1900s Kennel Clubs were formed to maintain breed standards, record pedigrees and issue rules for breed shows, and today they still impose strict restrictions on dog registration. For a dog to be an official member of breed the ancestors of each dog must be registered member as well (12).

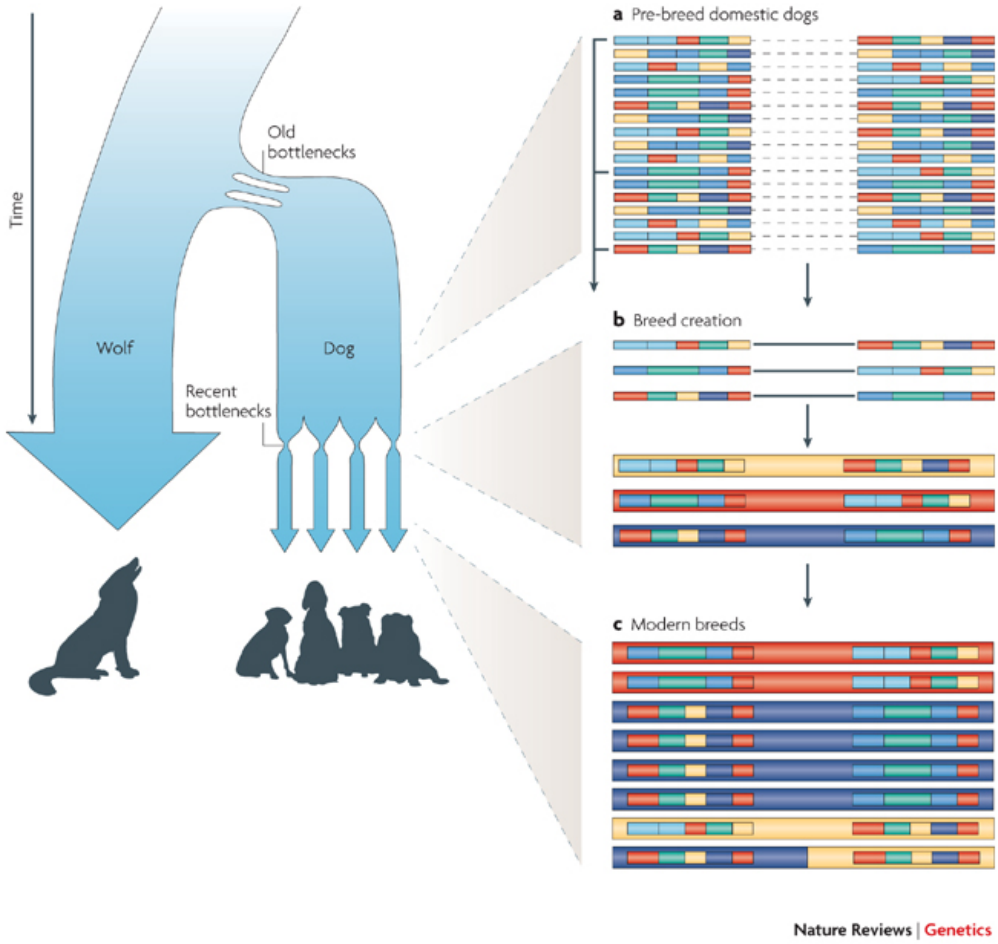

Figure 2 Haplotype structure of the dog

Two population bottlenecks in dog population history, one old and one recent, shaped haplotype structure in modern dog breeds. In a) the dog population had short-range LD and with the creation of modern breeds b) a small subset of chromosomes was selected from the pool of domestic dogs creating a long-range LD and since the breed creation took place not long a ago these long-range patterns have not yet been broken down by recombination and is found in the modern dog breeds c) (9). Reused with permission from Nature Reviews Genetics.

Widespread use of a popular sire has contributed to a decrease in genetic diversity and increasing the probability of identity by descent of undesirable alleles in his descendants (11). The strict breeding practices together with newer bottlenecks represented by fluctuations in popularity of the breed and catastrophes, such as war and economic depression, have further reduced the genetic variation within breeds and allele frequency divergence among them (13). Consequently, purebred dogs are members of closed breeding populations which receive little genetic variation beyond from what existed in the original founders (13-15). The process of creating dog breeds has led to an accumulation of disease risk alleles within certain breeds and an excess of hereditary diseases. Recessive diseases especially, are therefore common in purebred dogs (9,16).

Challenges of pedigree dog health, behavior and welfare

The creation of dog breeds and the strict breeding practices made sure that desirable features have been rigidly retained by inbreeding within closed familial lines. At the same time, as a consequence of reduced genetic variation, also undesirable disease-associated mutations/alleles can increase in frequency within the breed. Nearly 400 disorders that are caused or suspected to be caused by a genetic mechanism have been identified in purebred dogs (17). Some inherited disorders have thus shown to have a higher prevalence in many purebred dogs compared with non-purebred dogs. Breeding practices resulting in increase in homozygosity can therefore result in unnecessary suffering due to pain, disability, disease and behavioral problems (18).

Behavioral problems have an erosive effect on the bond between the dog and its owner, and is a common reason for relinquishment, it is estimated that they account for 10-15% of all euthanasias of dogs and cats in North America (19,20). In a study from the United States, at least one behavioral reason was recorded for 40% of relinquished dogs and behavioral reasons accounted for 27% of single-reason canine relinquishments (21). Behavioral reasons were given for approximately 11% of relinquished dogs from three animal shelters in Australia (22). In a study of 1,644 dogs referred to a behavior clinic, anxiety disorders and phobias were the second most common presenting complaint, only preceded by aggression (23).

The fast advances in canine genetics with genome-wide sequencing technologies and development of new diagnostic DNA-tests have also further increased the knowledge of inherited disorders, and there is increasing focus on how to implement new knowledge to improve the overall health of purebred dogs. The solution is not straightforward and involve a range of different strategies. Breeding strategies with screening schemes have shown to be successful in reducing the prevalence of inherited disorders and improving the health in certain breeds, e.g. hip- and elbow dysplasia (24). Such phenotyping selection; e.g. scoring of radiographs to detect and evaluate hip dysplasia have contributed to an improved genetic trend in many breeds, however selection intensities may be weak (24). Genomic selection is a method using genome-wide typing of marker genotypes in phenotypically scored animals to detect a subset of markers in LD with the disease (25). The use of such markers panels improve the estimates of the true breeding values, and has the potential to improve breeding progress. Breeding values may also be estimated for animals without phenotypic information. A potential advantage of genomic selection is that the genomic breeding values would be corrected for environmental influences (26). Testing and screening programs are vital to understand both the prevalence and susceptibility to developing disease, and creating breeding strategies with the aim of significantly reducing inherited disorders. DNA tests for disease causing mutation(s) will be most informative and effective for disease management. These must be combined with current screening schemes, pedigree information and genomic selection in order to maximize the impact in significantly reducing the number of inherited disorders and improving the overall health in purebred dogs (27).

The dog genome

The dog was the fourth mammal to have its genome sequenced, with a high-quality draft, with 7.5X coverage version released in July 2004 (1). It consists of 38 acrocentric, autosomal chromosomes and two sex chromosomes; a large sub-metacentric X chromosome and a small metacentric Y chromosome (28)(Figure 3). Humans have 22 autosomal chromosomes and two sex chromosomes, but these are larger than the canine chromosomes (29). Approximately 94% of the dog genome lies in regions of conserved synteny with humans (1). The 7.5X boxer genome that was released in 2004, was compared to a 1.5X poodle genome (30) and partial sequencing comparison of another nine dog breeds, resulting in a dense single nucleotide polymorphism (SNP) map containing more than 2.5 million SNPs (1).

The SNPs are evenly distributed across the canine genome and highly polymorphic across breeds. SNP arrays have been designed specifically for the dog genome; the most dense comprising >170,000 SNPs (Affymetrix, Santa Clara, CA, USA; Illumina, San Diego, CA, USA).

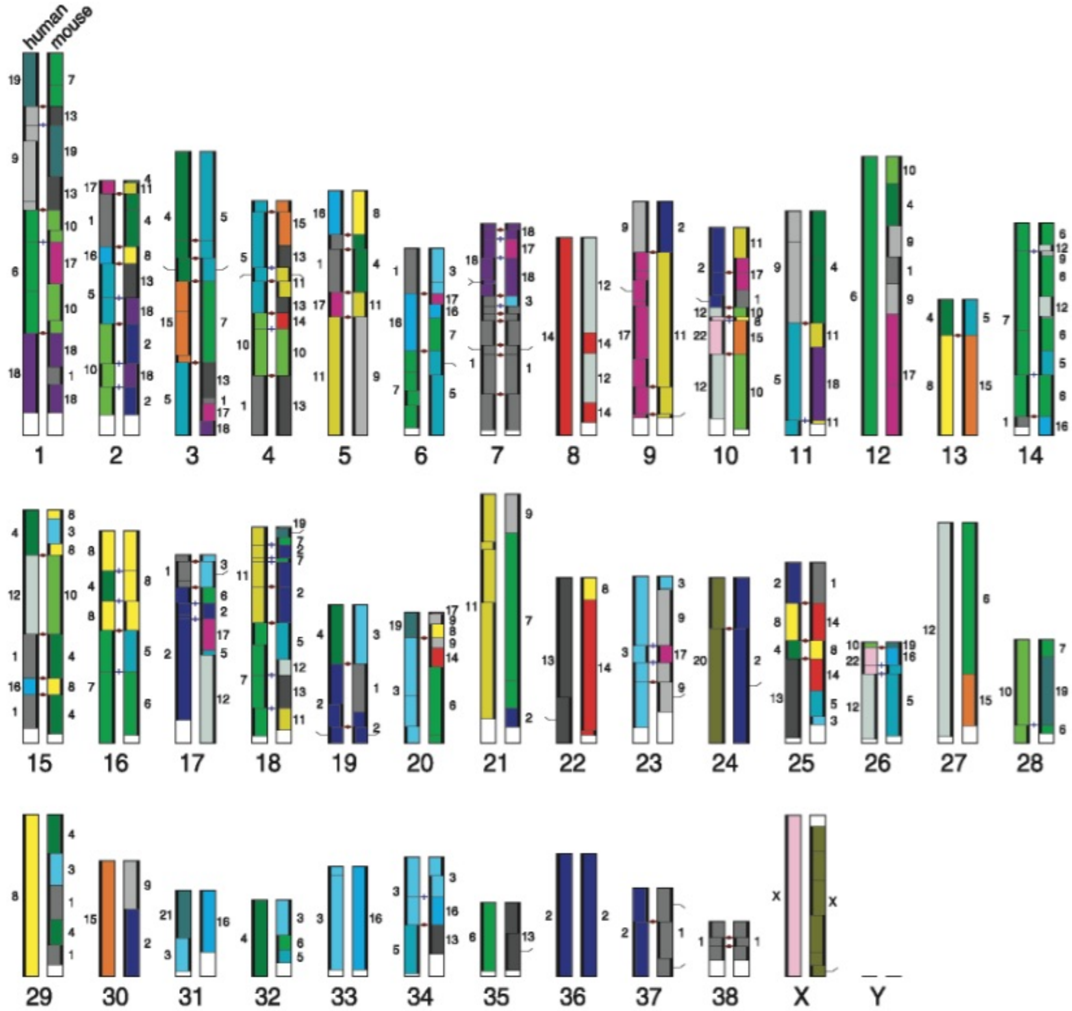

Figure 3 The dog chromosomes

A comparative map of the dog chromosomes. Each dog chromosome is represented twice, and compared to either human (left) or mouse (right) genomic segments. Map positions in dog increase from bottom to top along each dog chromosome. Distinct segments of conserved synteny between mouse and human are depicted by variously colored and numbered blocks corresponding to the 22 autosomes and X in human and to the 19 autosomes and X in mouse (30). Reused with the permission from Science.

The dog as a model organism

Animal models for human diseases have been extensively used, and have contributed significantly to the understanding of human hereditary diseases and development of improved treatment regimens. The many strains of the laboratory mouse has been the primary model (31), but as a model for more complex human disease the mouse has significant limitations (9). The dog however, has unique potentials in providing new insights into genetic disease, and have several advantages as a model organism. Diseases in dogs occur spontaneously during their life, as in humans, and include many common diseases like cancers, autoimmune diseases, heart disease, eye disease, diabetes, epilepsy and also psychiatric/behavioral disorders (12,32-35). The population structure of the dog is also advantageous, being the most physically diverse domesticated species (11). Each of the breeds we see today is defined by specific behavioral and physical characteristics that have been driven to exceptionally high frequency by population bottlenecks and strong artificial selection (9). This process has led to unintended consequences on the health of pure-bred dogs, with high rates of specific diseases in certain breeds and thus a lower genetic heterogeneity for disease genes is consequently seen. In humans, family history is one of the strongest risk factors for nearly all diseases (36), and the high prevalence of particular diseases in some breeds suggests a strong heritable component. The substantially increased risks in particular breeds suggests that just a few loci are involved, each with a strong effect, while there in human genetic disorders may be a high locus and allelic heterogeneity. The dog genome is less diverged from the human than the mouse genome, and have approximately the same number of genes as humans (1).

In addition to the many advantages that lie in the structure of the canid genome, the pet dog also shares living space with humans, possibly minimizing a potential effect of difference in environment. This is in contrast to other model animals, which live in a strictly controlled setting in laboratory facilities. Dedicated owners regard their dogs as part of the family, and dogs routinely receive medical treatment for many common diseases such as cancer, diabetes and epilepsy. With a lifespan that is much shorter than humans, diseases manifest at an earlier age, and typically run their course within a few years. Clinical trials are therefore of considerably shorter duration than in humans (37), and could provide useful testing ground for novel therapies.

In summary, the genetic similarity to humans, high number of naturally occurring hereditary diseases, unique population structure and shared environment, the purebred dog has emerged as a powerful model for study of diseases (31).

Behavioral genetics

The aforementioned processes behind the domestication has left the dog population divided into different breeds with an astounding degree of morphological and behavioral diversity. Many of the breed-specific behaviors, such as hunting, herding, guarding and pointing, will persist even in the absence of training or motivation, and thus are likely to be controlled in some part at the genetic level (38). Dogs also show differences in temperament, compulsive disorders, anxiety level, social behavior, aggression and more (32,39,40). Behavioral traits are complex, and determined by both genetic and environmental factors (41).

One of the most influential work on dog behavioral genetics started in 1945 by John Paul Scott and John L. Fuller, who collected data for several years. Their objective was to compare different breeds of dogs under environmentally similar conditions, so that any behavioral differences could be attributed to genetics rather than genetics and environment (41). Their work culminated in the publication of "Genetics and the Social Behavior of the Dog" in 1965, with the hypothesis that genetic effects act on specific behavioral traits (42). Another behavioral experiment spanning over three decades, studied the genetics of nervousness in English pointers (43,44). Two selection lines of dogs were established; one line exhibited extreme responses to noise, avoidance of humans, trembling and catatonia, while the other was a control line with stable temperament. Offspring produced from crosses between the two lines were similar to the nervous line and Murphree suggested that the nervous behaviors were inherited in an autosomal dominant matter (45,46). Beside the dog, foxes have been used in behavioral genetic research. Silver foxes (*Vulpes vulpes*) have been bred for over 50 years at the Institute for Cytology and Genetics (ICG) in Novosibirsk, Russia. Starting in 1959, Dmitry Belyaev selectively bred foxes for tame behavior towards humans, which resulted in a strain of foxes that showed high levels of sociable behavior towards humans, as well as a strain that was highly aggressive towards people (38,47-49).

Heritability of behavioral traits

Heritability calculation is a quantitative approach to understand the genetic contribution to canine behavior (50-53). The basic premise of quantitative genetics is that, if the relationships between individuals in a population are known, useful inferences about the inheritance of traits for which phenotypic data are available can be made without explicit knowledge of the genetic loci involved (54). Heritability (h^2) is the proportion of the total phenotypic variance that is attributable only to the additive genetic variance, and not to the variance from effects of dam or environment, and range from 0 to 1 (55). Traits with a heritability >0.4 are considered highly heritable (56). Recent studies have assessed heritability of behavior in working or pet populations, and estimates for some of the most studied behaviors can be found in Table 1. A study of four guiding dog breeds and their respective crosses, were tested for fearful reactions to various stimuli, found fearfulness to have a heritability of 0.5 (53). A study of Labrador retrievers and German shepherd dogs found heritabilities ranging from 0.14 for hardness to 0.38 for affability (willingness of the dog to approach humans) in the German shepherds, and from 0.03 for affability to 0.56 for gun shyness in the Labrador retrievers (57). Human-directed social behavior in research beagles have shown to have significant heritability, estimated to 0.23 (58). Another study of nearly 3,500 German shepherds, investigated seven traits: self-confidence, nerve stability, temperament, hardness, sharpness, defense drive and fighting drive, found heritabilites between 0.09 (sharpness) and 0.24 (reaction to gunfire) (59). Dogs have also been intensively bred to show behaviors such as pointing, nose work, retrieval, tracking and searching. One study found moderate to high heritabilities for different hunting traits (60). Other studies of hunting traits have found more moderate heritability estimates (0.006-0.183) in English setters and 0.01-0.15 in Finnish hounds (61,62).

Studies using the dog mentality assessment (DMA), where the results are condensed into five underlying personality traits; playfulness, curiosity/fearlessness, chase-proneness, sociability and aggressiveness, found heritabilities ranging from 0.14 (aggressiveness) to 0.25 (playfulness) in Rough collies (63). DMA data from nearly 6,000 German shepherd dogs found direct heritability estimates between 0.09 and 0.23, highest for playfulness and curiosity/fearlessness (64). Another study of DMA tested dogs found heritability of the personality trait shyness-boldness estimated to 0.25 in German shepherds and Rottweilers (51). Puppy testing has also been used to calculate heritabilities, and one study including German shepherd puppies found the highest estimates on the score of groups tug of war, activity and contact (0.42-0.53) (65). A behavioral test study of Hovawart puppies, found estimated heritabilites for the traits (contact, acoustic and optimal influences, prey drive, appearance assessment and temperament) ranging from 0.02 to 0.13 (66).

Heritability estimates pertain only to the population studied and can vary greatly between studies of the same traits, which is illustrated in Table 1, but can help change the frequency of a condition in a population of dogs (67). The estimates are however, a useful guide in breeding programs and the higher the heritability, the more gain will be made by selection (68).

Table 1 Heritability estimates for some of the most studied behavioral traits in dogs

	h^2	Breeds
Aggression	0.20-0.99	English cocker spaniel, Golden retriever
Fearfulness	0.05-0.88	Labrador retriever, German shepherd, Boxer, Kelpie, Rough collie
Herding	0.03-0.30	Border collie
Hunting	0.06-0.80	English setter, Finnish hound, German short haired pointer, German wire haired pointer, Griffon, Large munsterlander, Pudelpointer

Aggression: (69-71), Fearfulness: (50,72), Herding: (73,74), Hunting: (61,62,75)

Phenotyping

A phenotype is an observed characteristic of an individual that results from the combined effects of genotype and environment (76). Defining a behavioral phenotype is challenging as there is no specific physical characteristics and no clinical diagnostic approach, like blood tests or medical imaging as used in other diseases. However, phenotype is key to understanding genetic associations and with the use of rigorous criteria, behavioral diagnoses can provide associations between behaviors, pathology and environment (67). Phenotyping must be valid, reliable, sensitive and as objective as possible to be useful for genetic analysis (68). Measuring a behavioral phenotype include methods like battery testing, observational studies or owner reports as in questionnaires.

Numerous behavioral tests are applied to dogs. One of the most commonly used is battery testing where the core goal is to document dogs' reactions to specific stimuli by presenting various stimuli one at a time to a canine subject, and record its reaction (40). One example is the Dog Mentality Assessment (DMA), a standardized behavioral test used by the Swedish Working Dog Association, to test thousands of Swedish dogs each year (77). The test consists of 10 separate subtests; social contact, play, chase, passive situation, distance-play, sudden appearance, metallic noise, ghosts and gunshot. Comparing the test results with owner questionnaire responses it appears to reliably measure playfulness, sociability, curiosity/fearlessness and the boldness-shyness personality dimension. The DMA seems to be useful in predicting behavioral problems that are related to social and non-social fear, but not in predicting other potential behavioral problems (78). The Puppy Profiling assessment (PPA) is a puppy test developed by The Guide Dogs for the Blind Association in the United Kingdom (79). It was developed to be feasible, standardized and its criterion validity has been assessed under the framework for the development of behavioral tests for dogs (80). A study which analyzed the results of a pilot PPA study, showed that 5 of the 11 PPA stimuli showed some association with later success in guide dog training, and three stimuli could be usefully combined in a logistic regression model of success in training. However, adjustment to the scoring protocol were recommended (81). Factor analysis, or principle component analysis (PCA) is frequently utilized on behavioral tests to define a behavioral phenotype of interest, and these methods reduce a large number of behaviors assessed from a test to a smaller set of factors (34). Collected performance data needs to be adjusted for known environmental factors and a disadvantage is that factor analysis does not discriminate common genetic elements from common environmental elements (34,68). Studies of Finnish hunting dogs found that weather conditions and the month the trial was held, significantly affected performance. The authors attributed the low repeatability and estimated heritabilites of most hunting traits to the large effect environmental variation had on the results (82,83).

Owner-based questionnaires have been used in many behavioral studies. The owner is intimately familiar with the dog, and have the advantage to make an assessment of behaviors over time at home compared to a one-time event in an unfamiliar environment, which is common in battery testing. The Canine Behavioral Assessment and Research Questionnaire (C-BARQ) is a validated questionnaire that has been used in several studies (70,84-87). Owners assess either frequency or severity of situations using a 5-point ordinal scale. A study of aggressive behavior in the Golden retriever found that the C-BARQ was a more useful instrument for phenotyping than an aggression test (70). One study aiming specifically at using a questionnaire to identify the most fearful dogs to dogs not showing fear for gene mapping purposes, found excellent external validity with good repeatability for their questionnaire, suggesting that questionnaire can serve as an accurate and reliable phenotyping tool for fearfulness in dogs (88). Owner reported questionnaires may have a low reliability, due to a high number of assessors with different skills and interest to objectively describe the dog. One way to avoid this could be to ask the owners to select which response their dog show in specific circumstances to measure the reactivity, severity and intensity of the reaction in a more objective manner, Overall and colleagues have developed a questionnaire in this format (39).

CANINE ANXIETY

Fear, anxiety and phobia

The fear response is a normal and self-protecting behavior which can enable the dog to escape potential dangerous situations, but may, in some cases become inappropriate and negatively impact the dog's welfare. The terms anxiety and fear are often used interchangeably, but they have different definitions. Both are considered emotional responses to aversive stimuli, and are adaptive to enable avoidance of a perceived or anticipated threatening stimulus (Figure 4) (89,90). Anxiety is the emotional state elicited when animals are exposed to situations where there may be a threat (91,92). In other words, the dog shows signs of anxiety to a situation or stimuli which might occur, but the anxiety may be displayed in the absence of an identifiable stimulus as well (93). Fear is an adaptive response to the presence of stimuli considered to potentially be dangerous, and can be operationally defined as the collection of behaviors that occur in direct response to threat (94). The fear response allows the dog to avoid dangerous situations and increase chances of survival. Phobia is a sudden, excessive and profound fear. The phobic symptoms persist after the stimuli are removed or have disappeared, and the phobic reaction may occur in the absence of the trigger. Phobias are not an adaptive response, and interfere with normal functioning (95).

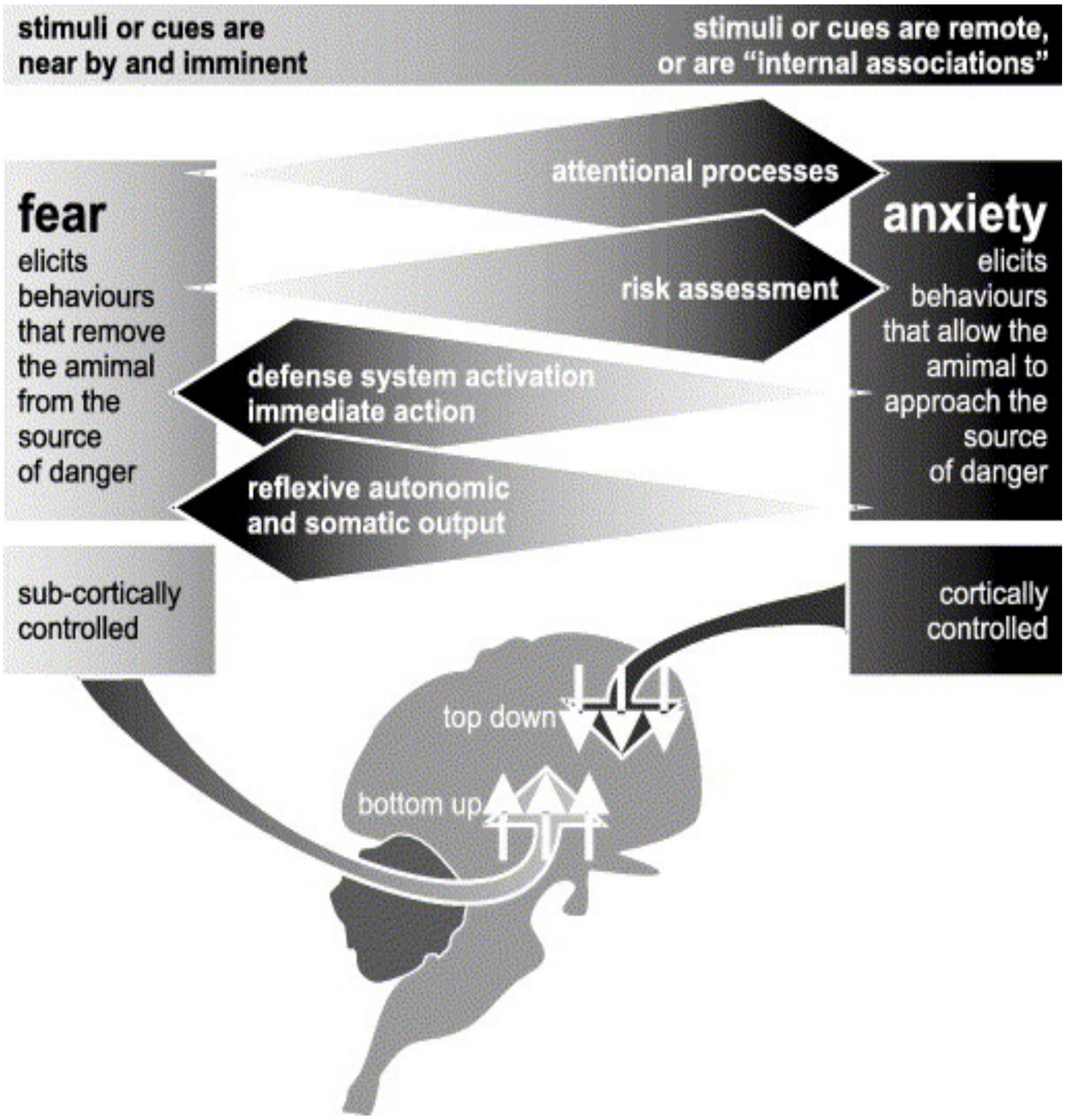

Figure 4 Distinction between fear and anxiety

Based on the proximity of the (perceived) threatening stimuli or cues and the level of cortical control (subcortical vs. cortical). Fear: the primary locus of control appears to be subcortical structures such as the amygdala that activates hypothalamic nuclei, that activate the sympathetic autonomic nervous system and the hypothalamic-pituary-adrenal axis, in parallel, higher brain regions such as hippocampus and the cerebral cortex may be activated ("bottom-up"). Anxiety: the frontal cortex is the primary locus of control; it processes the perceived threat cognitively and is able to modulate and steer lower levels of neuronal processing ("top-down") (89). Reused with permission from The Veterinary Journal, Elsevier.

On the physiological level, when an animal experiences anxiety, fear, or stress, both the sympathetic system and the hypothalamic-pituitary-adrenal (HPA) axis are stimulated, so that the body can respond to the threat (96,97). The sympathetic system releases adrenal steroids (noradrenaline and adrenaline) from the subcortical areas of the brain and adrenal gland, resulting in an increase in heart rate, blood pressure, respiratory rate, and vasoconstriction in internal organs (98). Several parts of the brain are involved in the fear response, with the amygdala playing a central role (Figure 5) (99-104). Dysregulation of fear pathways appears to be important in manifestation of the clinical signs associated with anxiety disorders. This dysregulation involves alterations in the activity of a number of neurotransmitters (93). Neurotransmitters are signaling molecules released from one neuron, through the synapses, in order to bind to receptors of the next neuron, and thus transfer the signal within the nervous system. There are a wide variety of neurotransmitters; serotonin, dopamine, acetylcholine, noradrenaline, adrenaline, gamma-aminobutyric acid (GABA) and glutamate representing some of them (105). The neurotransmitters and their receptors are central in behavioral research, and consists of a variety of proteins and ion channels, each coded for by a number of specific genes. The various regulation mechanisms and genetic variation make each of them a potential site for behavioral modification (106-111). The importance of neurotransmitters in behavioral modulation can be found in the science of psychopharmacology, where nearly all drugs target molecules related to neurotransmission (112). Anxiety disorders in humans have traditionally been treated with benzodiazepines (targeting e.g. GABA), selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs) blocking the reuptake of serotonin, but also monoamine oxidase inhibitors (MAOIs) (113).

Fearfulness in dogs can be categorized based on the object and the situation into social and non-social fearfulness. The social category includes settings with unfamiliar people and dogs, and the non-social fear category involves fear of different objects such as new situations, loud noises, heights and slippery/shiny floors (114).

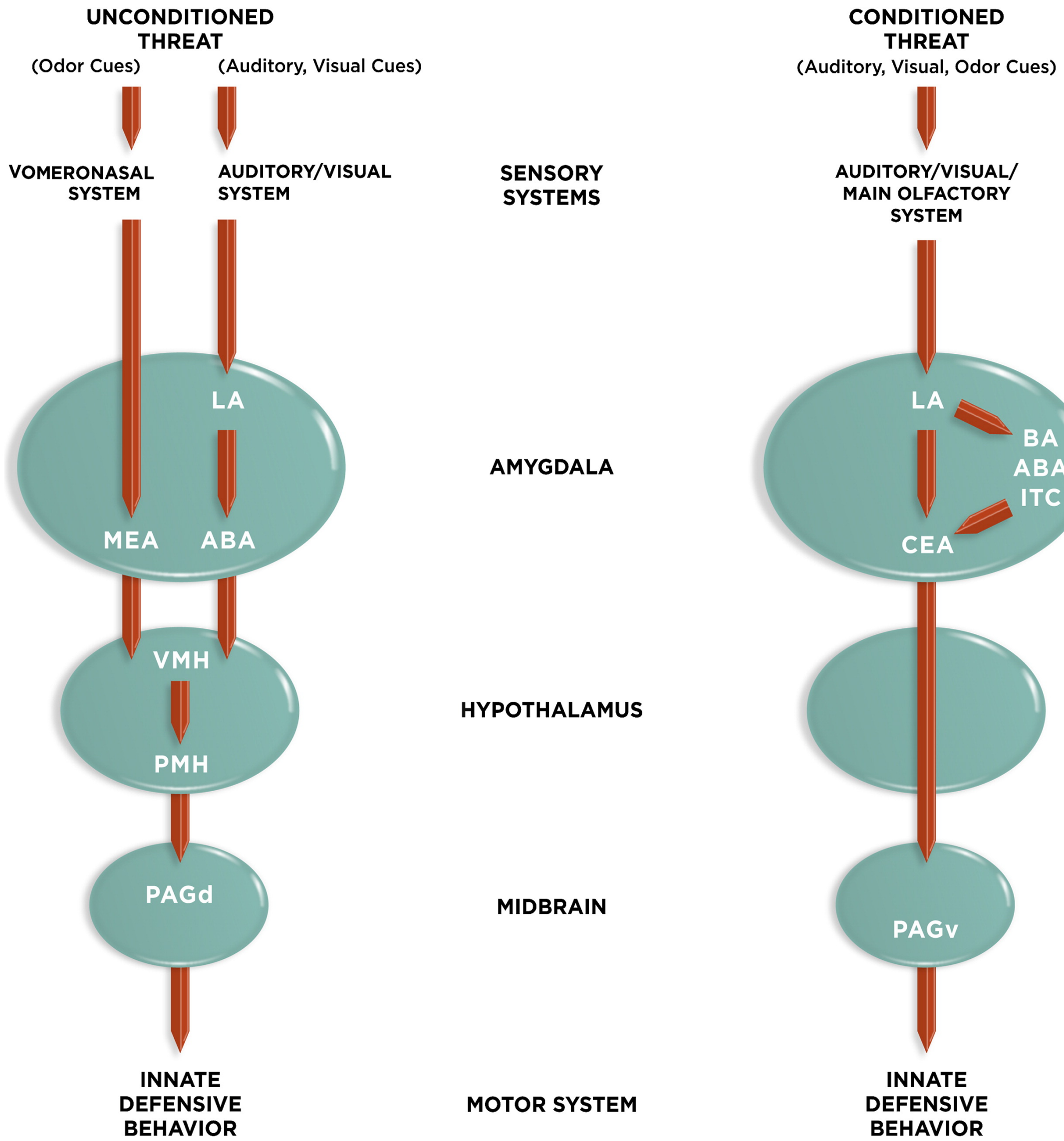

Figure 5 Circuits underlying defense reactions elicited by unconditioned (unlearned) and conditioned (learned) threats

ABA-accessory basal amygdala, BA- basal amygdala, CEA-central amygdala, LA-lateral amygdala, LH-lateral hypothalamus, MEA- medial amygdala, NAcc- nucleus accumbens, VMH- ventromedial hypothalamus, PAGd-dorsal periaqueductal gray region, PAGv-ventral periaqueductal gray region, PMH-premammilary nucleus of the hypothalamus (104). Reused with the permission from Neuron, Elsevier.

Behavior problems related to fear and anxiety are common in the domestic dog and can include generalized anxiety disorders, phobias, separation anxiety, noise sensitivity and fear-related aggression (115-117).

Separation anxiety in dogs

Separation anxiety (SA) is one of the most common canine behavioral problems (116,118,119). Studies have shown that separation anxiety accounts for 15% of canine behavioral cases seen by general practitioners, and up to 20-40% of cases seen by behaviorists (120,121). Separation anxiety is a welfare problem, and there is evidence that the stress of living with fear or anxiety disorders may strongly impact welfare, and can have negative effects on health and lifespan of the dog (122).

It has been postulated that separation anxiety could be an extension of a distress response from separation associated with a highly social state (123). The dog is a highly social species and exhibits attachment behaviors that serve to maintain social contact and bonds between adult individuals, as well as contact between parents and offspring (93). Domestication and selective breeding along with early socialization have contributed to increasingly affectionate, socially dependent, and infantilized dogs, which might be predisposed to excessive owner attachment and intolerance to be left alone (93,121,124).

Dogs with separation anxiety show distressed responses to being left alone, or being separated from the owner (115). Dogs may engage in a range of different behaviors when they experience separation anxiety, such as vocalization, destruction, elimination of urine or stools, anorexia, drooling, attempts to escape and (behavioral) depression (125,126). Vocalization, elimination and destruction being the most commonly reported behaviors (115). Separation anxiety can occur alone or together with other anxiety disorders. One study showed that the probability that a dog with separation anxiety also had sound sensitivities was 63%, and vice versa that the probability that a dog with sound sensitivities had separation anxiety was 88% (115).

Noise sensitivities in dogs

Noise sensitivity is another common behavioral problem in dogs (115,127). One survey including 383 dog owners reported that almost half (49%, n=188) of the owners stated that their dog was fearful of loud noises (128). In an online survey including more than 3,500 dog owners, 2,577 owners reported having a dog with noise sensitivity (129). A survey including veterinary practitioners in Spain, found that noise phobia was the fifth most frequent canine behavioral problem (130). Incidence data from behavior clinics may underestimate the underlying prevalence, since only a small number of dog owners are likely to seek specialist help. A study from New Zealand reported that only 15.8% of the owners with dogs that displayed a fearful response in situations with fireworks sought professional treatment (131). A Danish study found that owners of dogs with shooting phobia were less inclined to seek help with the behavior than owners of dogs with separation anxiety (132). Wells et al. reported that 68.3% of dogs purchased from an animal rescue shelter showed undesirable behavior within the first month and the majority, 53.4%, specified fearfulness as the major problem behavior (133). Behavioral responses of dogs with noise sensitivity can be extreme in nature, and it may represent a serious welfare issue for the dog.

Dogs can be fearful of many different noises, but the most commonly reported are fear of thunderstorms, fireworks, gunshots and engine noises (127,134). A study found that fireworks was the most commonly reported noise aversion, followed by thunderstorms and gunshots (129). Dogs with noise sensitivity may show a range of signs, including restlessness/shaking/trembling, pacing, increased startle response, increased vigilance, hiding, panting, drooling, destructiveness, defecation, urination, vocalization, withdrawal, self-mutilation, loss of appetence, freezing, vomiting, expression of anal sacs, owner-seeking and yawning (135).

GENETIC MAPPING STRATEGIES OF COMPLEX DISEASES

Many diseases are influenced by a combination of genetic and environmental factors. In simple recessive/Mendelian inherited disorders, usually one causative mutation leads to disease. In complex diseases, the disease phenotype is caused by the accumulated effect of several individual genetic variants with low penetrance, each contributing to increased risk (136) (Figure 6). Environmental factors might also modify the effect of genetic risk factors in complex diseases. There are several methods for identifying the genes underlying hereditary diseases. The most commonly used are discussed below.

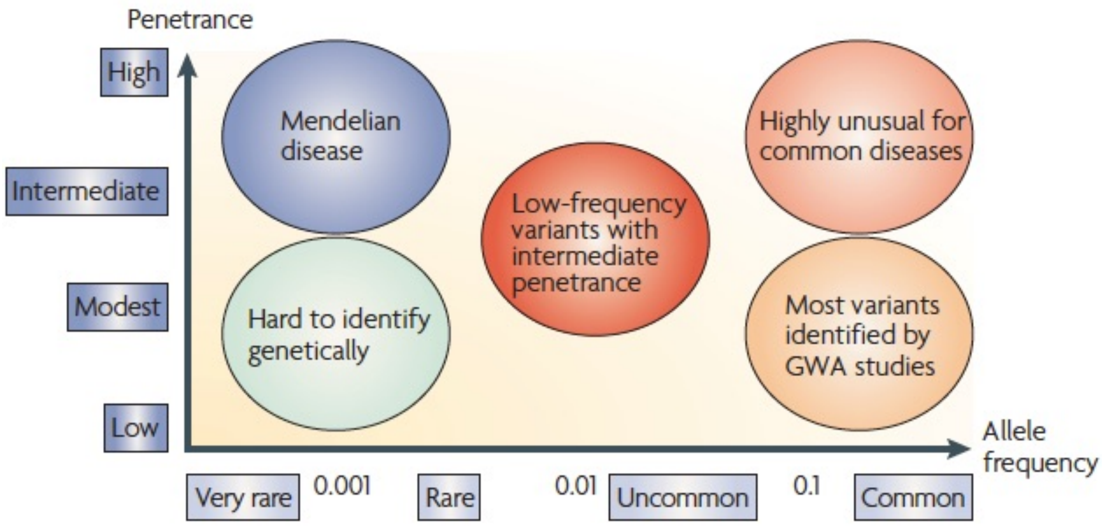

Figure 6 Low frequency variants and disease susceptibility

The relationship between the frequency of the causative alleles and the penetrance effect of them. The diseases studied in GWAS are thus caused by common alleles with a small effect size, or penetrance, whereas Mendelian disease are caused by rare alleles with large effect size. Figure modified from McCarthy et al. (136). Reused with the permission from Nature Reviews Genetics.

Candidate gene approach

Candidate gene studies are based on *a priori* knowledge of potential genes that might be involved in the pathogenesis of the phenotype to be studied. Candidates might be genes that have been previously associated with the phenotype in other breeds or species. Typically, genotype and allele frequencies of the candidate genes are investigated and compared between cases and controls. In candidate gene studies of behavioral traits, for example; genes found to be linked to anxiety in humans can be studied in dogs (137). This approach has for the most part concentrated on those genes involved in the regulation of common neurotransmitters. An example is the study of involvement of the serotoninergic and dopaminergic system in various canine behavioral disorders (109,138-140). An advantage of this method is the relatively low cost. However, only genes already known to have an effect on the disease phenotype are investigated by this approach, impeding the discovery of novel mutations.

Linkage studies

Linkage analysis is used to map genetic loci associated with a phenotype of interest by the use of related individuals (families) and genetic markers (e.g. microsatellites). The genetic markers are used to identify regions that co-segregate with the phenotype, and therefore may harbor the causative gene or mutation that are linked to the disease phenotype. Linkage studies can be applied to both major gene disorders and complex diseases, and have proven successful in identifying genes for Mendelian diseases. However, the effect sizes of the causative alleles of complex diseases are expected to be small. Mapping genes associated with complex diseases using linkage analysis requires very large family materials (141). Linkage studies will therefore not always be applicable for mapping genes associated with complex traits, because the effect of individual causal variants of complex traits is too small to be detected via co-segregation within pedigrees (142). After identification of an associated gene region by linkage analysis, the genes in the region must be further evaluated by e.g. candidate gene study, fine mapping or re-sequencing.

Genome-wide association studies

In genome-wide association studies (GWAS), usually unrelated individuals (cases and controls) are studied. Unrelated individuals are easier to sample than related ones, and the advantage of using unrelated individuals is that identified regions will be smaller due to more recombinations around a disease causing mutation. There is, however, a risk of genetic heterogeneity in the collected cases compared to sampling of related individuals. In dogs it is therefore recommended to perform GWAS within one single dog breed or two closely related breeds with the same phenotype. Whereas linkage studies exploit co-segregation within families, GWAS is based on the LD at the population level. SNP markers are studied in GWAS and this greatly increases the number of available markers, and the coverage of the genome is denser. Genotyping microarray platforms are used to type the cases and controls for a large number of predefined genetic polymorphisms (SNPs), based on the SNP map constructed for the canine genome (1). Such microarrays or SNP chips have been developed for use in dogs as well (Affymetrix, Santa Clara, CA, USA; Illumina, San Diego, CA, USA). The genotype frequencies in GWAS are compared between cases and controls, and allelic association with the disease is established using chi-square tests (141). Population stratification can however lead to false positive associations and needs to be taken into account (143). A common method of testing the sample population is by creating a multidimensional scaling (MDS) plot (Figure 7). Using randomly selected autosomal markers, a genomic kinship matrix weighted by allele frequencies is computed. The genomic kinship matrix is then used to perform multidimensional scaling (MDS) which projects genetic distance between individuals in two dimensions. As a large number of SNPs are tested for one or several phenotypes in the dataset, multiple hypothesis testing correction is required to avoid report of false associations. Permutation testing or Bonferroni correction are most commonly used for the multiple hypothesis correction of the statistical GWAS results. The result is often displayed as Manhattan plots, which has obtained its name due to the resemblance of the Manhattan skyline (Figure 8). The Manhattan plot is a type of scatter plot used to display data with a large number of data-points. The position of the SNP is on the X-axis arranged by chromosome number, and on the Y-axis the association of the SNP to the tested phenotype is displayed as -log10(p-value), thus the higher on the Y-axis the lower the p-value (Figure 8).

The use of a high number of SNP markers results in a very good coverage of the genome in GWAS, and for several years GWAS has been the method of choice for detection of genetic variants associated with complex diseases. GWAS rely on LD between the genotyped SNPs and unknown casual variants, and as the SNPs on the arrays are selected for being common, GWAS are powered to detect association with casual variants that are relatively frequent in the population. Thus, GWAS are especially useful for detecting common genetic variants that are associated with common diseases (142). Alleles identified through GWAS are typically not causative, but rather in LD with true causative variants (144).

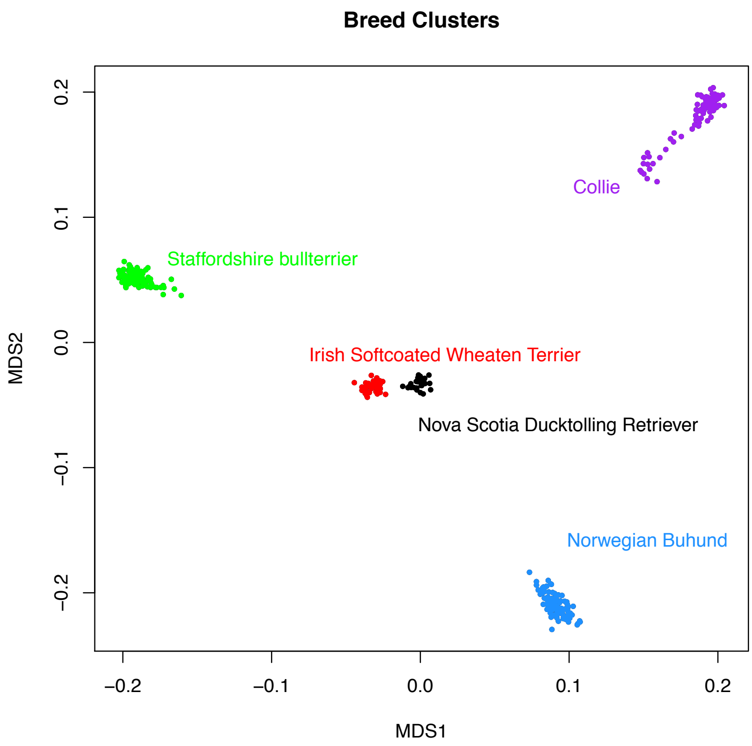

Figure 7 Multidimensional scaling (MDS) plot

An example of population stratification illustrated with a MDS plot, the breeds are five genetically separated populations. The MDS plot projects genetic distance between individuals in two dimensions (from paper III).

Even though GWAS have provided valuable information about the genetic basis of disease, DNA sequence variants identified by GWAS typically explain only a small fraction of the heritability of most complex traits. From the early studies another problem emerged, described as the "missing heritability problem". Although studies have discovered many (>1,200) variants associated with common diseases and traits, the variants typically appear to explain only a minority of the heritability (145). A good example of this is the heritability of height, which has been estimated to be 80-90% heritable, but when large GWAS was performed, the variants they found accounted for little more than 5% of height's heritability (146). This "missing heritability" has shifted the focus from the hypothesis that common variants cause common diseases, towards rare variants exerting larger effect sizes (147). Other causes of complex diseases could be joint action of numerous loci of small effect, genetic heterogeneity, epistasis (interactions between two or more genes to control a phenotype), gene-environment interactions, epigenetic effects, and regulation of microRNAs and mRNAs (148,149).

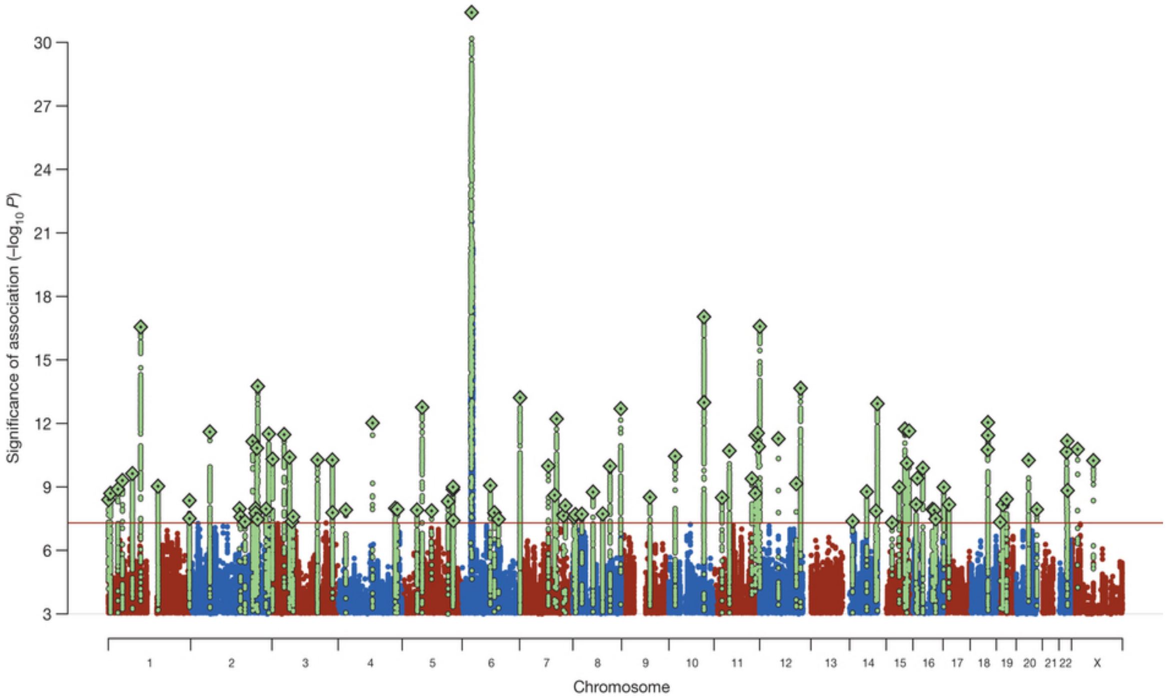

Figure 8 Manhattan plot of schizophrenia meta-analysis

The position of the SNP, each dot signifies one SNP, is on the X-axis arranged by chromosome number, and on the Y-axis the association of the SNP to the tested phenotype is displayed as -log10(p-value), thus the higher on the Y-axis the lower the p-value. The red line is the genome-wide significance level (5×10^{-8}) (150). Reused with the permission from Nature.

Next-generation DNA sequencing analysis

GWAS have the strength to decipher novel mechanisms in the pathogenesis of complex phenotypes where effect sizes of the identified common variants usually explain only a minor fraction of the variability and heritability of the complex traits (148,151). Next-generation sequencing (NGS) offers the opportunity to sequence the entire exome or genome to elucidate the genetic etiology of complex traits. In DNA sequencing by NGS, multiple fragments of DNA are sequenced simultaneously and the output aligned to a reference genome (141). All DNA sequence variant of an individual genome, both common and rare, might be identified, with the exception of large copy-number variations (CNVs), and large duplicated regions. Both re-sequencing of already annotated regions/genes/genomes and *de novo* sequencing can be performed. The massive data produced by NGS presents a significant challenge for data storage, analyses and management solutions, and advanced bioinformatics tools are essential for the successful application of NGS technology (152).

The significant decrease in the cost of sequencing has allowed whole-genome or exome sequencing to be more frequently used as a method of analysis. Today many of the NGS studies focuses on genome re-sequencing to identify variants potentially accounting for the "missing heritability" problem observed in genetically complex traits. Promising progress has been made in the field of mental disorders, and identification of causative mutations using these new technologies will facilitate neurobiological studies of these complex traits (153,154).

EPIGENETICS

Epigenetic changes are heritable changes influencing on gene expression and includes mechanisms other than alterations in the DNA sequence. Epigenetic mechanisms may change the highly complex organization of DNA in a cell nucleus and include many types of histone and DNA modifications, as well as alterations in many types of non-histone proteins and non-coding RNAs (155). Some of the most common epigenetic effects involve methylation; a methyl group is tagged onto a region of the DNA that affects how, or whether it is transcribed. These modifications can occur sporadically as a result of e.g. normal aging or environmental exposures, or they can be heritable. As epigenetic regulation is vital for normal growth and development, deviations can lead to disease and has been found in e.g. autoimmune diseases and cancer in humans. Such mechanisms have been considered possible mediators of responses to environmental factors and may therefore play a certain role in the pathogenesis of psychiatric disorders as well (156). The ultimate goal of epigenetic studies of mental illness is to understand how genetic vulnerabilities interact with an individual's life experiences to establish stable changes at precise genetic loci, which then control the levels of gene expression or inducibility (155).

AIM OF THE STUDY

The overall aim of this thesis was to evaluate breed differences and prevalence of anxiety disorders, and to identify candidate genes and mutations associated with anxiety disorders in the dog.

Sub goals:

1. Characterize dogs with separation anxiety referred to a behavior clinic (paper I)
2. Identify and characterize breeds/dogs with high occurrence of anxiety disorders (paper II)
3. Study of genetic variations in specific anxiety related gene(s) in dog breeds with high and low incidence of anxiety disorders (paper III and IV)
4. Identify genomic regions associated with anxiety in dogs (paper III)

MATERIALS AND METHODS

ANIMALS

Genetic studies

In collaboration with breed clubs and owners, samples were collected through dog shows, home visits and owners sending blood samples or buccal swabs by mail. Samples from Irish softcoated wheaten terrier (ISWT, n=44) and Staffordshire bull terrier (SB, n=137) were collected in the time interval between 2008-2014. Blood samples from Nova Scotia duck tolling retriever (NSDTR, n=33) and Collie (n=98) were collected from local and national dog shows from 2011 to 2013 (paper III and IV). Samples from Norwegian Buhund (NB, n=108) were collected in collaboration with the Norwegian Buhund club at events/dog meetings (paper III). Poodles (n=29) and Havanese samples (n=158) were collected from dog shows and home visits in 2014 (paper IV). All samples were collected for research purposes, with owners' consent.

Epidemiological studies

Dogs included in paper I were all the dogs that visited a behavior clinic and were diagnosed with separation anxiety from April 2007 to August 2010 (n=215). Paper II consisted of 5,257 dogs, across 17 breeds, where the breed clubs had participated in a voluntarily online general health and behavior survey.

QUESTIONNAIRES

Owners of the dogs included in paper I filled in a questionnaire before the clinical consultation. The sections included: (i) Information about the owners: gender, geographical location and number of family members living with the dog; (ii) general information about the dog: age, gender, neuter status, breed; (iii) general information about the daily life including: where the dog was obtained, daily activity and feeding, rewards, training, punishment; (iv) occurrence of behaviors in different situations: when the dog was alone, behavior towards people, behavior towards other dogs, behavior in situations with loud noises and a specific section about elimination. Paper II was based on an extensive web survey of general health and behavior of the dogs. Items included in the study was the section concerning reactions to loud noises and fear responses in other situations in addition to general information about the dog such as age, gender, neuter status and breed. Questionnaires used for phenotype classification in paper III and IV were based on a five point scale, commonly used for measuring behavioral traits in dogs (157).

STATISTICAL ANALYSIS

Odds ratios (OR) were estimated for comparing difference between sex/neutering in paper I using a clinical research calculator (vassarstats.net) and to measure co-occurrence between noise aversions and fear responses in other situations in paper II. An analysis of variance (ANOVA) was performed to explore potential breed effects; comparing the means between breeds to determine if any of the means are significantly different from each other with the null hypothesis H_0: $\mu_1 = \mu_2 = \mu_3 = \ldots = \mu_k$, where μ is the group mean and k number of groups (breeds). If at least two group means were significantly different from each other, a Tukey post hoc test was performed to determine which specific groups differed from each other (paper II). Association between noise sensitivity (dependent variable), breed, sex and age (independent variables) was analyzed with an ordinal logistic regression model in paper II. Co-occurrence between the categories of noise aversions was measured with Spearman's correlation test (paper II). For paper II the analyses were performed with Stata statistical software (version 12.0). The genome-wide association analyses in paper III was performed using R v3.2.1 and GenABEL v1.8-0. In paper IV OR was estimated for the SNPs using a clinical research calculator.

GENETIC ANALYSIS

In paper III and IV, genomic DNA from the dogs was analyzed. The DNA was extracted, either from blood or saliva. Genomic DNA from blood was extracted from the EDTA tubes using E.Z.N.A blood DNA kit (Omega Bio-Tek, Norcross, GA, USA). The saliva samples were collected using swabs by Performagene™ from the PG-100 collection and DNA was extracted according to the manufacturer's recommendations. The genotyping of the SNPs in paper III was carried out using the Illumina 170k CanineHD Bead chip. Sequencing of the candidate genes in paper IV was performed following a standard Sanger method on an ABI 3500 XL DNA analyzer (Applied Biosystems, Life Technologies of Thermo Fisher Scientific), followed by manual inspection using the Sequencher software from Gene Codes Corporations.

SUMMARY OF THE PAPERS

Paper I

A descriptive study of 215 dogs diagnosed with separation anxiety

Clinical records of dogs visiting a behavioral clinic were used to study the behavior and background of dogs with separation anxiety (SA). 215 dogs (with SA) were included in the study, representing 22.6% of the patients seen during the 40 months the study covered (n=952). Male dogs comprised 60% (n=129) of the patients, and females 40% (n=86). Neutered dogs were more common in the clinical material compared to reference populations. More male dogs diagnosed with SA were neutered compared to female dogs with SA (28% n=37 vs. 8% n=7). Forty (18.5%) dogs were diagnosed with SA only, while 179 (82.8%) of the patients had other behavioral problems in addition to SA. The most common co-morbid diagnosis was noise sensitivities (43.7% n=94). Owners of the dogs presented for clinical evaluation most commonly reported vocalization, destruction and excessive motor activity (as signs of SA). Some breeds seem to have a higher incidence of separation anxiety than other breeds. The majority of the owners were families consisting of two adults or adults with children, and most of the owners had obtained their dog from a breeder as a puppy. Twenty-eight (13%) of the owners were women living alone and three (1.4%) being a man living alone.

Paper II

Noise sensitivity in 17 dog breeds: prevalence, breed risk and correlation with fear in other situations

A web-based survey was conducted to estimate the prevalence of noise sensitivity in 17 dog breeds in Norway (n=5,257), with major focus on noise from fireworks, loud noises (bang/gunshots), thunderstorms and heavy traffic. The study also investigated risk factors as well as correlation with some other fear responses. On average, approximately 23% of the dogs were reported to be fearful of noises. Fear in situations with fireworks had the highest frequency; situations with loud noises/gunshots, thunderstorms and heavy traffic following in decreasing order. Across the 17 breeds there were significant (p<0.01) differences in the frequencies of fearful dogs. Norwegian Buhund, Irish softcoated wheaten terrier and Lagotto romagnolo were the breeds that had the highest frequency of noise sensitivity, while Boxer, Chinese crested and Great dane had lower frequencies of fear created by noise. There was a significant trend of increasing fear with age. Response to fireworks, loud noises/gunshots and thunderstorms frequently co-occurred. Female dogs had higher odds of noise sensitivity compared to male dogs (OR=1.3 $p<0.001$), and neutered dogs had higher odds of being fearful of noises than intact dogs (OR=1.73 $p<0.001$). The dogs most fearful of noises also had higher odds of showing separation related behavior, being fearful in novel situations and required longer time to calm down after a stressful event, compared to dogs less fearful of noises.

Paper III

A genome-wide association study for noise sensitivity in 5 dog breeds

Noise sensitivity is a common problem in dogs and show significant differences in incidence between breeds. In this study, we searched for genetic factors that could be associated with fearfulness of loud noises. A genome-wide association study (GWAS) of over 400 dogs across five breeds (Collie, Irish softcoated wheaten terrier, Nova Scotia duck tolling retriever, Norwegian Buhund and Staffordshire bull terrier) was performed. The study showed large variations in allele frequencies between the breeds for many of the markers, but did not reveal significant genome-wide associations with anxiety. The study may however be underpowered due to potential genetic heterogeneity within and between breeds. Allele frequencies between the breeds were evaluated for the top SNPs from each GWA analysis and in an area around an anxiety candidate gene *DRD2,* and were found to vary greatly both within and between the breeds. In addition, many markers showed nearly a total fixation. The allele frequencies of the major allele, also showed opposite direction in cases and controls in some markers between breeds.

Paper IV

***DRD2* is associated with anxiety in some dog breeds**

Samples from five dog breeds (Collie, Irish softcoated wheaten terrier, Havanese, Nova Scotia duck tolling retriever and Standard poodle) with a total of 358 dogs, were studied for association of general anxiety and noise sensitivity to SNPs in the dopamine receptor gene D2. Dopamine is one of many neurotransmitters in the brain, and is known to influence behavior and mood. Both human and animal studies have implicated that the *DRD2* gene is involved in the development of depression and anxiety. The dogs were classified based on owner questionnaires or observational evaluation. Three of the breeds showed significant association ($p < 0.05$) with anxiety to the two synonymous SNPs in exon two in *DRD2.* The risk allele was the same in all three breed showing association: C in the first SNP and T in the second SNP.

RESULTS AND DISCUSSION

Anxiety is a complex trait involving several genetic risk factors. The genetics and etiology behind complex diseases such as behavioral traits largely remain elusive. The work in this thesis focused on describing the prevalence and difference in frequency of anxiety disorders between several breeds, the correlation between the various types of fear-related behavior problems and in the final part the main purpose was to identify regions and mutations in genes associated with anxiety disorders in the dog.

The epidemiological studies were conducted to get a better understanding of risk factors and breed differences in canine anxiety disorders, and to be able to select breeds with a high number of anxious dogs for the genetic studies. The dogs diagnosed with separation anxiety (paper I) included many breeds, and mixed breed dogs were most numerous in this material, followed by Cocker spaniel, Gordon setter, Schnauzer and Dachshund breeds. Even though the dogs included represented many breeds, we found that many of the top 10 SA breeds had a much lower rank in the number of registered puppies at the Norwegian Kennel Klub, i.e. they were not among the most popular breeds at the time of the study. The study of noise sensitivity (paper II) included 17 different breeds, and there was a statistical significant difference between the frequencies of fearful dogs between breeds. The same breeds showed a consistently high number of fearful dogs across all the categories of noise, and included Norwegian Buhund, Shiba inu, ISWT, Lagotto romagnolo and NSDTR. Breed was also found to be a risk factor for noise aversions in a study from the UK (117). The prevalence of noise sensitivity was found to be quite high, with an overall of fearful dogs of 23%. In paper II, an online questionnaire was the basis for the study and answered by the dogs' owners. This may underestimate the actual prevalence since an unexperienced owner may miss signs of fear in the dog or in the case were the signs are not easily recognized by owners as a sign of fear, e.g. decreased activity or salivation. Owner-based questionnaires may be sensitive to the owner's subjectivity and interpretation, but one of the most commonly used, the C-BARQ, has shown to be reliable and valid for assessing behavior and temperament traits in dogs (84). Gender differences were evaluated in both studies. In the SA study more males were diagnosed with SA than female dogs, and more male dogs with SA were neutered compared to female dogs. In the noise sensitivity study (paper II), female dogs had higher odds of being fearful to loud noises, and neutered dogs were more likely to be fearful than intact dogs. This could be a reflection of the population of dogs included in the two studies. In the SA study (paper I) male dogs represented 60% of the material, while in the noise sensitivity study the population consisted of slightly more females. However, further research is needed to investigate the relationship between fear responses and gender. In the noise sensitivity study there was found a significant trend of increasing fear with age, this is in accordance with other studies were dogs with noise sensitivities were observed to be older compared to control dogs (117,158). Some fear responses were found to co-occur in both studies. Over 80% of the dogs diagnosed with separation anxiety had other behavioral problems, and noise sensitivity was the most common co-morbid diagnosis. In the noise sensitivity study, the response to the different noises frequently co-occurred. The dogs most fearful of noises also had higher odds of showing separation related behavior, being fearful in novel situations and also required longer time to calm down after stressful events compared to dogs less fearful of noises.

The significant differences found between the breeds in the first studies indicates that risk alleles of anxiety genes may have been accumulated in specific breeds during selection. Samples from some of the breeds found to have high frequency of fearful dogs in the first studies were therefore used in the genetic studies in the second part of the work in this thesis. A GWAS was performed with the purpose of identifying regions and markers associated with noise sensitivity (paper III). This study included five breeds comprising over 400 dogs. In 2006, the first SNP microarray containing ~ 27000 SNPs (Affymetrix) for the canine genome was available, and it was estimated that genome-wide association studies could be efficiently performed to map autosomal recessive traits using 20 affected and 20 controls (159). Since then numerous studies have been conducted in dogs, several successful in identifying loci associated with the phenotype in question (160-165). Although progress has been slow in revealing genetic risk factors underlying behavioral traits, there has been identified SNPs segregating with the boldness-shyness axis in dogs (166,167). To date, only one study has achieved genome-wide statistical significance identifying a gene related to a behavioral disorder, linking the cadherin 2 gene (*CDH2*) to flank sucking in Doberman pinchers (168). In our study no significant genome-wide associations were identified, and the SNPs with the lowest p-values varied between the breeds, with no regions overlapping across the breeds. There was a great variation in allele frequencies both within breeds and between breeds, and some markers showed "opposite direction" in the cases and controls in several breeds. Research in humans suggests that rare polymorphisms may have significant population specific effects on phenotypes, and it is reasonable to believe that rare genetic variation within breeds may have important effect on phenotypes studied as well (34). The study confirms the challenge of using several breeds and that genetic heterogeneity between unrelated breeds will make it difficult to achieve genome-wide significance. The top SNPs in each breed were on different chromosomes and due to the complex genetic nature of anxiety, where many genes probably are involved, it is not expected that the same risk alleles are shared between unrelated breeds. In studies with limited sample sizes, it would probably be a better alternative to use SNPs within specific candidate genes to detect associations to behavior traits. This approach was chosen in the final study where there was found association of SNPs in the dopamine receptor gene *DRD2* to general anxiety and noise sensitivity in several breeds (paper IV). Dopamine is an important neurotransmitter in the brain and is involved in the regulation of behavior, the dopamine receptor D2 is found presynaptic and is part of the negative feedback cycle when dopamine levels are elevated. Human studies have implicated that the *DRD2* gene is involved in the development of depression and anxiety (169,170). Of the five breeds included, three showed significant association to either general anxiety or noise sensitivity with two SNPs in exon two. The allelic frequency varied between the breeds in this study as well, but the same risk allele was observed for the two markers in the breeds with significant association.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Canine anxiety is a common behavioral problem causing deleterious effects on the dog's health and welfare. Deciphering the genetic changes behind anxiety disorders has the potential to improve diagnosis and treatment in dogs, and might also provide useful insight to the pathogenesis of human anxiety disorders. The present work found significant differences between the frequency of fearfulness in the breeds studied, which points to genetic risk factors underlying behavioral traits such as anxiety. The GWAS performed in paper III confirmed that studies including several breeds with limited sample sizes may be suboptimal to detect genome-wide significant associations, and that candidate gene studies may be a better approach to explore genetic risk factors to complex traits such as behavior. An association to canine anxiety was found to SNPs in the dopamine receptor gene *DRD2* in several breeds in the candidate gene approach. Further studies including more individuals will increase the power of genome-wide association studies, making it possible to discover novel loci associated with behavioral traits. It will then be possible to search for the causative mutations located within these loci. However, the causative mutations may not be found in protein coding regions, and investigating promoter regions, various non-coding RNAs (microRNAs), duplications, copy number variations with whole-genome sequencing could provide new insight into genetics behind behavioral traits.

In the recent years there has been an increased focus on the high incidence of inherited diseases in purebred dogs, and a large number of genetic tests have been developed. Due to the complex genetic alterations underlying behavioral traits, a wide range of alleles and loci are expected to be involved in the pathogenesis of behavioral disorders. Identification of inherited risk factors for use in genetic testing, risk estimation and selection of the best breeding material may be important parts of a strategy to improve dogs' health, but should be used with care to avoid unfavorable negative correlations. As the knowledge increases, robust genetic tools will probably also be available to assist selection for complex diseases in order to assist in breeding healthier dogs and aiming at increased animal welfare.

The dog, with its unique population structure and the high similarities between human and canine diseases, provide unique opportunities for comparative studies within the field of medical genetics. The wide variety of behavior traits between breeds, and the presence of high risk breeds for specific behaviors should make the dog especially valuable as a model for behavior research. The dog is an excellent model for future studies on hereditary complex diseases to the benefit of both dogs and humans. The results from the present thesis are valuable for further use in the ongoing search for the understanding of the genetics underlying behavioral traits and disorders in the dog.

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A descriptive study of 215 dogs diagnosed with separation anxiety

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ABSTRACT

Clinical records of dogs visiting a behavioral clinic were used to study the behavior and background of dogs with separation anxiety (SA). 215 dogs (with SA) were included in the study, representing 22.6% of the patients seen during the 40 months the study covered ($n = 952$). Male dogs comprised 60% ($n = 129$) of the patients, and females 40% ($n = 86$). Neutered dogs were more common in the clinical material compared to reference populations. More male dogs diagnosed with SA were neutered compared to female dogs with SA (28% $n = 37$ vs. 8% $n = 7$). Forty dogs (18.5%) were diagnosed with SA only, while 179 (82.8%) of the patients had other behavioral problems in addition to SA. The most common co-morbid diagnosis was noise sensitivities (43.7% $n = 94$). Owners of the dogs presented for clinical evaluation most commonly reported vocalization, destruction and excessive motor activity (as signs of SA). Some breeds seem to have a higher incidence of separation anxiety than other breeds. The majority of the owners were families consisting of two adults or adults with children and most of the owners obtained their dog from a breeder as a puppy. Twenty-eight (14%) of the owners were women living alone and three (1.5%) being a man living alone.

1. Introduction

Behavioral problems are common in pet dogs (Gonzalez Martinez et al., 2011). Incidence of problems varies significantly between different populations/studies. A retrospective study of 1644 behavior cases in dogs showed that the most common problems were those involving aggression followed by anxiety-related conditions not involving aggression. Of the latter separation anxiety was the most common diagnosis (Bamberger and Houpt, 2006). Owners are most likely to notice and report behaviors that they do not like in their dog, without regard for whether these behaviors are abnormal or a problem for the dog (Overall, 2013a), this may also reflect why aggression is the most common behavior problem reported. A high proportion of Danish dog owners stated that their dog had one or more behavior problems (29% of 4359 dogs) (Rugbjerg et al., 2003). Salman et al. (2000) reported that at least one behavioral reason was recorded for 40% of the dogs and behavioral reasons accounted for 27% of single-reason canine relinquishments in the study including a total of 2230 dogs from 12 shelters and four regions in the United States (Salman et al., 2000). High prevalence rates of behavioral problems in both American and European dog populations (Bamberger and Houpt, 2006; Rugbjerg et al., 2003) are reflected in the number of dogs being relinquished to animal shelters due to behavioral problems (Salman et al., 2000) and dogs brought to animal clinics for

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euthanasia (Houpt et al., 1996; Miller et al., 1996). Several reports show that the most common reason for euthanasia and relinquishment is the dog's behavior (Gonzalez Martinez et al., 2011; Miller et al., 1996; Mondelli et al., 2004). One third of the dogs that are adopted from shelters are returned because of the dog's behavior (Shore, 2005) A Danish study found that behavioral problems was the third most common reason for euthanasia, only preceded by old age and cancer.

The high prevalence of behavior problems including anxiety and the negative influence on animal welfare supports that increased efforts should be invested to understand the background for these behaviors. Responses to fear and stress are the root of a wide range of behavioral problems in domestic dogs, canine separation anxiety being one of them.

Dogs with separation anxiety show distressed responses to being left alone or being separated from the owner (Overall et al., 2001). When dogs experience separation anxiety they may engage in a range of different behaviors; vocalization, destruction, elimination of urine or stools, anorexia, drooling, attempts to escape and (behavioral) depression (Horwitz, 2009). Vocalization, elimination and destruction are the most commonly reported behaviors (Overall et al., 2001; Sherman and Mills, 2008). As the signs are non-specific it is important to explore other anxiety-related behavioral problems in order to make the correct diagnosis. Separation anxiety can occur alone or together with other anxiety disorders. One study showed that the probability that a dog with separation anxiety also had sound sensitivities was 63% and vice versa that the probability that a dog with sound sensitivities had separation anxiety was 88% (Overall et al., 2001).

The causes of separation anxiety are multi-factorial (environmental and genetic) and the underlying motivations, proposed in the literature are fear, anxiety, over-attachment/hyper-attachment or lack of appropriate stimulation (Horwitz, 2009). Hyper-attachment includes following the owner from room to room, including wanting to follow the owner to the bathroom, wanting to sleep next to its owner and the dog being distressed when separated from the owner (Appleby and Pluijmakers, 2004). The importance of hyper-attachment is debated in the literature, and some findings suggest that separation anxiety may be due to a different attachment style between dogs with and without separation anxiety (Parthasarathy and Crowell-Davis, 2006). A study (from Australia) suggest that separation-related distress may not be purely attachment-based (McGreevy and Masters, 2008). Separation anxiety could also be caused by a more general state of anxiety, which is suppressed when the dog is in contact with the owner (Bradshaw et al., 2002). Another study clearly found factors associated with hyper-attachment to the owner to be significantly associated with separation anxiety (Flannigan and Dodman, 2001). Different findings (contradictory results) regarding the sex of dogs with separation anxiety have been reported, (McGreevy and Masters, 2008; Takeuchi et al., 2000) found that male dogs outnumbered female dogs and that male dogs had higher probability of elevated levels of separation-related distress. (McGreevy and Masters, 2008) also found that intact dogs showed a higher probability of high separation-related distress scores than neutered dogs, this is in contrast to the study by (Flannigan and Dodman, 2001) which found that sexually intact dogs were more than three times less likely to have separation anxiety as neutered dogs.

Several factors that could play a role in developing canine separation anxiety include periods of kennel housing, shelter housing, a history of long periods of being left alone, long periods with the owner without being left alone, the family moves to a new house/apartment and loss of another pet in the family (Sherman and Mills, 2008). A recent study showed that dogs obtained from pet stores where 30% to 60% more likely to have separation-related problems than dogs obtained from noncommercial breeders (McMillan et al., 2013). Dogs may also have a genetic predisposition to develop anxiety (Serpell, 1995).

The objective of this study is to describe characteristics of a group of dogs diagnosed with separation anxiety in order to better understand the potential genetic and environmental effects important for the etiology of anxiety.

2. Materials and methods

2.1. Dogs surveyed

The study was based on clinical records (retrospectively) from a behavior clinic in Norway. All the dogs that visited the clinic and were diagnosed with separation anxiety ($n = 215$, 22.9% of the total number of patients) from April 2007 to August 2010 were included in the study. Most of the dogs in this study were obtained from a breeder as a puppy (which is the most common way to obtain a purebred dog in Norway) while 43 dogs (21.3%) had previous owner(s) before the current owner. The neutered dogs with SA included in this study were already neutered when the owners contacted the behavior clinic.

2.2. Classification of behavior

The study is based on clinical observation of the dog from the behavior clinic, owner interviews and questionnaires. One ethologist (GL) with a master in companion animal behavior counselling made all the diagnoses during clinical consultation and was based on discussions with the owner and review of a questionnaire that was filled in before consultation. The diagnosis was made on the basis of a behavioral history and the exclusion of diagnostic differentials; and the conditions for a diagnosis of separation anxiety in this study was that the dog showed behavioral signs of distress in the absence of the owner or when the dog could not gain access to the owner when they are at home. Inclusion criteria were that the dogs showed consistent signs of destruction, vocalization and/or elimination when the owner was absent. The dogs included also showed anxiety/distress at the time of the owner's departure and/or exaggerated greeting behavior and showed signs of strong attachment to one or more family members.

The questionnaire includes basic questions about the dog and its background and sections with more detailed questions about behavior in different situations. The

Table 1
Top 10 breeds diagnosed with separation anxiety at the behavior clinic compared to top 10 breeds of the overall population from the behavior clinic, the most popular breeds registered at the Norwegian Kennel Club (NKC) and the rank of breeds visiting a larger small animal clinic.

Behavior clinic (SA)	Behavior clinic (all patients)	NKC 2005–2010	Small animal clinic
Mixed breed	Mixed breed	German shepherd	Mixed breed
Cocker spaniel	German shepherd	Norwegian elkhound	Labrador retriever
Gordon setter	Cocker spaniel	Golden retriever	German shepherd
Schnauzer	Jack Russell terrier	Cavalier king charles spaniel	English setter
Dachshund, long-haired	Border collie	Border collie	Rottweiler
Jack Russell terrier	Golden retriever	English setter	Boxer
Dachshund, short-haired	Rottweiler	Gordon setter	Jack Russell terrier
German shepherd	English springer spaniel	Labrador retriever	Standard poodle
Tibetan spaniel	Flat-coated retriever	Tibetan spaniel	Flat-coated retriever
Rhodesian ridgeback	Dachshund, long-haired	Irish setter	Cavalier king charles spaniel

sections included: (i) Information about the owners: gender, geographical location and number of family members living with the dog; (ii) general information about the dog: age, gender, neuter status, breed; (iii) general information about the daily life including: where the dog was obtained, daily activity and feeding, rewards, training, punishment; (iv) occurrence of behaviors in different situations: when the dog is alone, behavior toward people, behavior toward other dogs, behavior in situations with loud noises and a specific section about elimination.

2.3. Descriptive analysis

This study focused on the section of the questionnaire related to the “home-alone” situation. Breed, sex and neuter status were included to be able to study potential breed differences, sex-distribution and frequency of neutering. The breed distribution in patients were compared to three reference populations; all patients from the behavior clinic in the 40 months the study covered, the national register of the Norwegian Kennel Club and data from one large small animal clinic in the region to study breed risk of specific behaviors. The results of the dogs with SA regarding sex and neuter status are compared to the overall population from the behavior clinic during the same time period and general health surveys of 7 dog breeds ($n = 2120$). (The descriptive results are presented as frequency tables/figures and odds ratios.)

3. Results

3.1. Breed distribution

Mixed breed dogs were the most frequently $n = 28$ (13%) recorded group with separation anxiety; the rest comprised 93 different dog breeds. The other frequently recorded breeds are found in Table 1.

Table 1 gives an overview of the breeds that are most frequently diagnosed with separation anxiety in the behavior clinic as well as the overall breed distribution of all patients from the behavior clinic. For comparison the top ten breeds from NKC and a small animal clinic were added. Cocker spaniel is the breed that is most frequently diagnosed with separation anxiety in the behavior clinic, even though the breed represents only 1.8% of the dogs recorded in NKC and is not found in the top 10 of breeds registered at the NKC in the years 2005–2010. Another example is in the group of Schnauzer (pooled), which represents 2.8% of the total patients at the behavior clinic, but only 0.3% of the total dog population (NKC). Separation anxiety is also frequently recorded in Dachshunds. Some breeds seem to be overrepresented at the behavior clinic, compared to the number in the total dog population in Norway (NKC).

3.2. Sex/neutering

In our study 20% (Table 2) of the dogs were neutered which is about twice the frequency compared to dogs in general health surveys in 7 breeds (Table 2). The dogs with separation anxiety also showed a higher frequency of neutering compared to all the dogs from the behavior clinic where only 80 (8.4%) were neutered (Table 2) (reasons for neutering, see Fig. 1 and Section 3.3). Of the SA-dogs, seven (8%) of the females and 37 (28%) of the male dogs were neutered, while in the 7 reference breeds, 81 (9%) male dogs and 122 (10%) female dogs were neutered. The odds ratio of neutering for males compared to females is 4.65 ($p < 0.0002$) in SA dogs, while in the overall behavior clinic population the odds ratio of being a male dog and neutered is 2.94 times higher than female dogs ($p = 0.0004$). For comparison in the 7 reference breeds the frequency of neutering was lower in males (OR 0.90).

Of the SA-dogs 60% were male dogs, while in the 7 reference breeds the frequency of males were 42%. 603 (63.3%) of all the dogs from the behavior clinic were males and 349 (36.7%) females, of these 66 (11%) of the males were neutered versus 14 (4%) of the females (Table 2). This indicates there is a higher frequency of male dogs with separation anxiety compared to females in this study

Table 2
Sex distribution, number of intact and neutered dogs.

Sex	Neutered; *n* (%)	Intact; *n* (%)	Total (%)
Dogs with separation anxiety			
Male	37 (29)	92 (71)	129 (60)
Female	7 (8)	81 (92)	86 (40)
Total	44 (20)	171 (80)	215
All patients from the behavior clinic			
Male	66 (11)	537 (89)	603 (63)
Female	14 (4)	335 (96)	349 (37)
Total	80 (8.4)	872 (91.6)	952
7 dog breeds from general health surveys			
Male	81 (9)	815 (91)	896 (42)
Female	122 (10)	1101 (90)	1223 (58)
Total	203 (9.5)	1916 (90.5)	2119

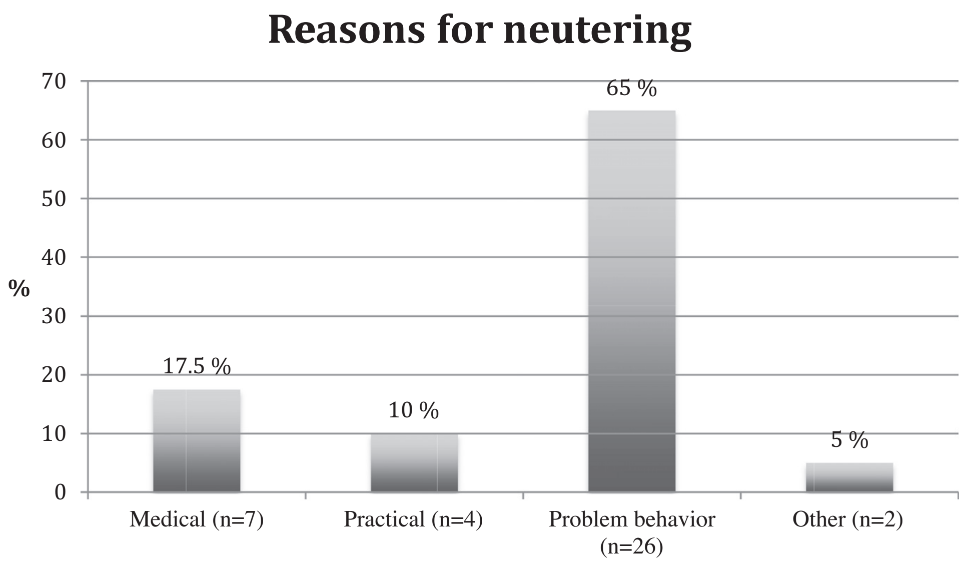

Fig. 1. Reasons given by owners for neutering their dog (in percent), total $n = 39$.

($p < 0.01$). The sex fraction in dog litters should be close to 0.5 and there is no local data that support a skewed sex-distribution with a higher proportion of males in dog populations. Data from local general health surveys have a higher frequency of responders of female dogs as shown in Table 2. Mean age of the dogs when they were neutered was 2.1 years ($n = 35$) and mean age when the dogs were taken for consultation at the behavior clinic for the first time was 2.7 years

3.3. Reasons for neutering

The major reason (65%) for neutering of the 44 dogs with SA was according to the owner behavior problems, other reasons are shown in Fig. 1. The specific behavior reason for neutering (specified in 28 dogs) was sexual behavior (e.g. mounting, reduce high activity of male dogs when close to female dogs in heat) ($n = 8$, 15.3%), marking/house soiling ($n = 2$, 3.8%), aggression ($n = 5$, 9.4%), hyperactivity/wanted a calmer dog ($n = 11$, 20.8%) and separation anxiety/general anxiety ($n = 2$, 3.8%). Most of the owners reported that there had been no change in behavior ($n = 15$, 28.3%) after neutering the dog, 11.3% ($n = 6$) reported that the neutering had a desired effect on the behavior and 15.1% ($n = 8$) said that the behavior in question became worse after neutering.

3.4. Owner information/risk factors

The recording of environmental factors was limited in this study, but we looked closer into some records of the family situation to the dogs (Fig. 2). The majority of the dogs lived in a family consisting of two adults or adults with children (71%). Women living alone represented 14% versus men living alone 1.5%. This might indicate that there could be a difference in risk of separation anxiety depending on the gender/family situation of owners. In the general population in Norway between 2005 and 2011 the percentage of men and females living alone is relatively equal, 9% men and 8.7% women.

3.5. Behavior of the dogs with separation anxiety

Fig. 3 shows the distribution of some of the reported behaviors of the dogs in this study that are associated with separation anxiety. The most common complaint of the owner is vocalization followed by destruction and stress/excessive activity.

3.6. Other behavior problems

Of the 215 dogs with separation anxiety included in this study, 18.1% ($n = 39$) had no other reported behavioral problem. The rest of the dogs (81.9%, $n = 175$) had one or more additional behavioral problems. Ninety-four (43.7%) of the dogs were diagnosed with fear of noises in addition to the separation anxiety. Aggression in different forms was the second largest group of observed behaviors of the SA-dogs in this study. Seventy (32.6%) were classified with fear-related aggression, 4.2% ($n = 9$) showed aggression related to aversive painful interactions, 5.1% ($n = 11$) displayed territorial aggression and 2.3% ($n = 5$) showed aggression related to resources (e.g. food, bones and toys). Additional behavior problems were compulsive behaviors, (4.2%, $n = 9$), and coprophagia (2.3%, $n = 5$). As some of the dogs had multiple problems the numbers will not add up to 215.

3.7. Sleeping habits

The questionnaire includes questions about how the dog acts around the owner when at home and where the dog sleeps during daytime and at night. Over half of the dogs (51.3%, $n = 103$) sleep in the bed with the owner during the night. Results from the general health surveys show that 15.8% ($n = 336$) of the dogs regularly (“always” or “very often”) sleep in the owner’s bed while 40.4% ($n = 857$) never sleeps in the bed with the owner.

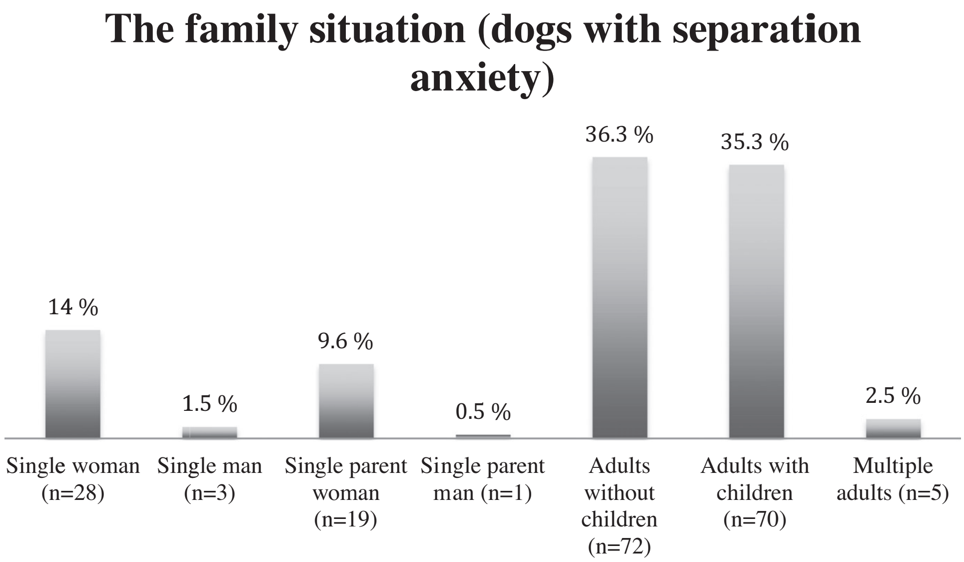

Fig. 2. The family situation of the dog with separation anxiety, total $n = 198$.

3.8. *Behavior when the owner leaves and returns*

When the owner prepares to leave the house the majority of the SA dogs show signs of anxiety and distress ($n = 126$, 75%). Twenty dogs (11.8%) showed no specific behavior when the owner departs. A small number ($n = 4$, 2.4%) showed signs of owner-directed aggression when the owner is about to leave. When the owner returns 91.5% ($n = 140$) of the dogs diagnosed with separation anxiety shows an excessive greeting ritual like jumping and vocalization.

3.9. *Where the dogs are when the owner is away*

The majority of the dogs have access to only parts of the house when the owners are away ($n = 67$, 37.2%), while 31.1% ($n = 56$) is in a cage or constrained in a small area in other ways. Some dogs (25.6%, $n = 46$) have access to the entire house. Only 3.9% ($n = 7$) are never left home alone. The rest of the dogs ($n = 4$, 2.2%) are outside when left home alone.

4. Discussion

The study describes some of the main characteristics of dogs diagnosed with separation anxiety at a behavior clinic in Norway. The number of living dogs for each breed in the population is unknown and in population studies in dogs it is therefore a challenge to get good estimates of the reference population. To get an expression of the relative number of each breed we have used nationwide registration data from the Norwegian kennel club and a large small

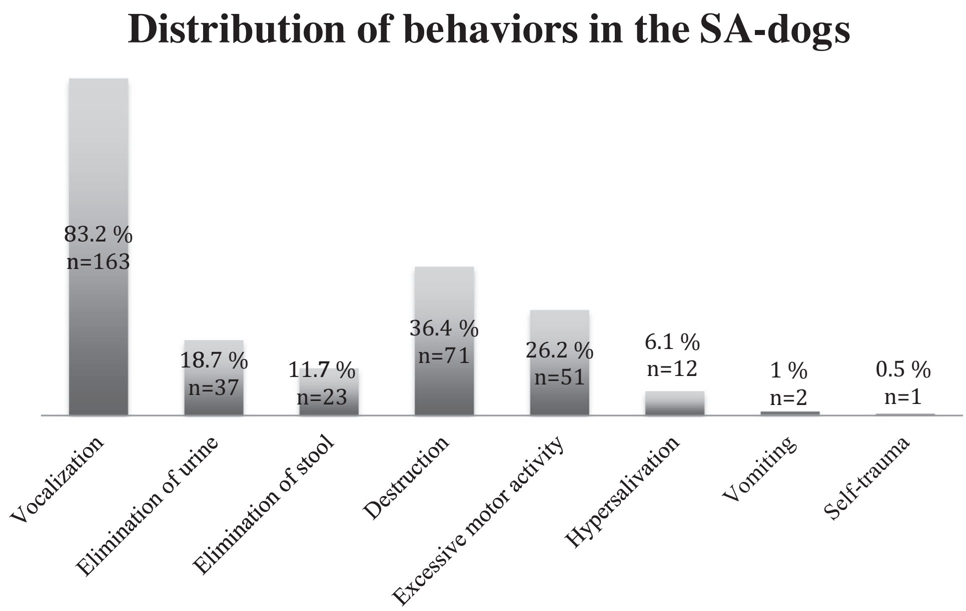

Fig. 3. Distribution of behaviors (expressed) in dogs diagnosed with separation anxiety, total $n = 198$.

animal clinic in the region. The comparison of the rank of the various breeds with the rank of the breeds from the behavioral clinic gave an indication of relative breed prevalence of separation anxiety.

It seems that some breeds are more likely to be diagnosed with separation anxiety than others. This difference in breed prevalence may indicate an accumulation of risk-genes/alleles in some breeds. Even though the 215 dogs represent many breeds, many of the top 10 SA-breeds represented at the behavior clinic have a much lower rank in the number of registered puppies at the NKC (i.e. not of the most popular breeds at the time of the study). We believe that the information from the number of registered dogs combined with data from larger small animal clinics gives a relatively good impression of the breed distribution. Three of the breeds with highest frequency of SA; Cocker spaniel, Schnauzer (group) and Dachshund are not recorded among the top 10 breeds neither in the NKC nor in the small animal clinic. (Flannigan and Dodman, 2001) found in their study that the subsequent order of breed incidence in the separation anxiety group was Golden Retrievers, English Springer Spaniels and English Cocker Spaniels; purebred dogs were predominant in both affected and control group, but overall more mixed-breed dogs had separation anxiety than did purebred dogs, although the difference was not significant. Even if the numbers from the veterinary clinic might be influenced by differences in breed disease frequencies, all records are included; e.g. routine health care visits and vaccinations, and should be less influenced by breed. There could also be a bias if the owners of certain breeds are more likely to take their dog to a behavior consultant, but we have no data to support this. This difference in breed frequency of SA could indicate that some breeds are genetically predisposed to this behavior problem, but this needs to be investigated further.

The sex distribution is somewhat skewed in this study, almost 60% of SA dogs are males and 40% females, which is similar to findings from other studies on SA. Overall et al. (Overall et al., 2001) reported 57% males and 43% females in their study of frequency of nonspecific clinical signs in dogs with separation anxiety, thunderstorm phobia and noise phobia. This is also in accordance to an Australian study where they found that male dogs had a higher probability of exhibiting elevated separation-related distress scores than females (McGreevy and Masters, 2008). The sex distribution from the general health surveys in 7 dog breeds shows a higher frequency of females (58% females and 42% males). Since there is no data to support that there are more males in the general dog population we believe that our data support an increased risk of SA in males (a skewed distribution of 60% males in a population of 215 dogs is significant at $p < 0.05$). This is in contrast to a previous study where they found that sex of the dog was not associated with separation anxiety (Flannigan and Dodman, 2001). Our results also show a higher frequency of neutered dogs with SA (20%) compared to the general population (10%) and to all the patients from the behavior clinic (8.4%). If we expect that the frequency of neutering due to disease is equal we would expect that the difference in neutering associated with behavior problems would be bigger. 29% of the males with separation anxiety are neutered versus only 8% of the females; the male/female neutering odds is 4.65 ($p < 0.01$) in dogs with separation anxiety. A neuter frequency due to “general preference” in the general dog population in Norway is relatively low (prohibited), and dogs are first and foremost neutered due to specific health and behavior reasons. This represents a situation different from that in some other countries where neutering often is done routinely at a young age. Again we might expect that more females than males are neutered to disease-related problems (pseudo-pregnancy, ovarian hysterectomy), supporting that more males may have been neutered in an attempt to improve behavior. Compared to the numbers from the health surveys there was about the same percentage of neutering in both females and males (9.9% and 9% respectively). The majority of the owners in this study (65%) give that the reason for neutering was due to the behavior of the dog and only 11.3% said that the neutering had a desired effect on the behavior. Behavior problems are one of the primary reasons for owners to request neutering of male dogs even if there is no support of an effect of neutering on behavior problems. Previous studies have showed that only sexually dimorphic behaviors like urine-marking, mounting and roaming are usually reduced by neutering (Maarschalkerweerd et al., 1997; Neilson et al., 1997). To neuter dogs with behavior problems that are not related to sexual behavior (like fearfulness or anxiety) would therefore not be expected to give a positive outcome.

Vocalization was the most common behavior reported by the owners of the dogs diagnosed with SA. Destruction and excessive motor activity was the second and third most common. This is similar to results found in previous studies (Overall et al., 2001; Flannigan and Dodman, 2001; Palestrini et al., 2010). Bradshaw et al. (2002) also found in their cross-sectional study that barking was the most common symptom, followed by destructive behavior and howling. As all these symptoms are non-specific it is important to consider other etiological factors. Other reasons for vocalization includes stimuli coming from outside the house, territorial displays and fears, but in the inclusion criteria we have used excessive vocalization which is much stronger/more frequent than when the owner is at home/within sight of the dog. Other reasons for destruction could be over-activity, playful behavior, territorial behavior, fearful stimuli and noise sensitivities. Excessive activity could also be a product of playful behavior, reaction to arousing stimuli and over-activity, but again according to our inclusion criteria these behaviors have been much worse when the owner is not at home.

Excessive motor activity can be difficult to perceive when the owner is absent. Videotapes will be helpful to verify the diagnosis, but were unfortunately not available for the dogs in this retrospective study. Separation-related behaviors could be the consequence of different underlying states (discomfort, fear or anxiety), direct observation with videotapes would help to differentiate between these states and treatment would be more targeted (Palestrini et al., 2010). Videotapes of the dogs when left alone can also be of great value during behavioral therapy to assess the efficacy of the therapy program (Blackwell et al., 2006). However, most owners are able to detect extensive changes in behavior when they are about to leave, with increased activity and barking; and combined with other behaviors mentioned in the inclusion criteria, we believe that the dogs would correctly be classified with separation anxiety. Motor activity can also be present when the owner is virtually absent from the dog, meaning that the owner is unavailable to the dog being in another room with a closed door, in this case it is easier for the owner to observe the activity of the dog. The motor activity may also be evident prior to the owner's departure. Since the dogs in this study were not video taped when home alone the results about excessive motor activity must be interpreted with care. Even if obvious symptoms such as destruction and elimination of urine/stool are easily recognized there is a risk that the frequency of separation anxiety is underestimated. In milder cases with no destruction, or in cases where excessive vocalization is not heard (no neighbor complains) the separation anxiety may remain unobserved.

The majority of the dogs (43.7%, $n = 94$) included in this study were also diagnosed with noise sensitivities. Even though the etiology for the development of separation anxiety for the most part remains elusive there is evidence that if dogs react to or are fearful of noises in a repeatable manner, they may have increased incidence of separation anxiety and/or other anxiety-related conditions (Overall, 2013b). Similar results were found in a study including 141 dogs diagnosed with separation anxiety and/or noise phobia, the probability that the dog had storm phobia given that he had separation anxiety was 61% (Overall et al., 2001). (Flannigan and Dodman, 2001) also found that almost half of the dogs with separation anxiety were fearful of noises, whereas less than third of the control group had a similar fear. This indicates that different forms of anxiety may have a partial overlapping etiology and that some dogs may be at risk for several types of anxiety.

Most of the SA dogs in this study live in a home with multiple owners (two or more adults with or without children), however a rather large part of the dogs live in a household with only one adult (13% single women and 1.4% single men). This is in accordance to a study by (Flannigan and Dodman, 2001) which found that dogs from a home with a single adult human were about 2.5 times as likely to have separation anxiety as dogs from homes with multiple persons. McGreevy and Masters (2008) found a significant relationship between an increased number of adult female humans in the household and elevated levels of separation-related distress. We show that the majority of the single owners were women living alone. An explanation for this could be that female owners establish a different relationship to dogs compared to men or that women have a lower threshold to seek help. This study is not conclusive about gender of the owner being associated with separation anxiety and this needs to be investigated further.

The importance of hyper-attachment is debated in the literature, and some findings suggest that separation anxiety may be due to a different attachment style between dogs with and without separation anxiety (Parthasarathy and Crowell-Davis, 2006). And dogs included in this study also showed a strong attachment to one or more family members. Even so our data is not conclusive on hyper-attachment. We do however observe that there is a skewed distribution of some owner groups and a high percentage of dogs that are sleeping in the bed of the owner. If this is a random effect, a functional association or an expression of extended caretaking of problem-dogs is unknown.

5. Conclusion

This study supports previous findings of characteristics of dogs diagnosed with separation anxiety. In summary more males than females are diagnosed with SA and there is a tendency that more males were neutered because of behavior problems. There seem to be a breed-specific tendency indicating an accumulation of risk-genes/alleles in some breeds, but further efforts including genetic studies should be performed to establish a certain connection between specific breeds and a separation anxiety diagnosis.

Conflict of interest statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Noise sensitivity in 17 dog breeds: Prevalence, breed risk and correlation with fear in other situations

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ABSTRACT

A web-based survey was conducted to estimate prevalence of noise sensitivity in 17 dog breeds in Norway ($n = 5257$). Major focus was on noise from fireworks, loud noises (bang/gunshots), thunderstorms and heavy traffic. The study also investigated risk factors as well as correlation with some other fear responses. On average approximately 23% of the dogs were reported to be fearful of noises. Fear in situations with fireworks had the highest frequency; situations with loud noises/gunshots, thunderstorms and heavy traffic following in decreasing order. Across the 17 breeds there was significant ($p < 0.01$) differences in the frequencies of fearful dogs. Norwegian Buhund, Irish Soft Coated Wheaten Terrier and Lagotto Romagnolo were breeds that had the highest frequency of noise sensitivity while Boxer, Chinese Crested and Great Dane had lower frequencies of fear created by noise. There was a significant trend of increasing fear with older age. Response to fireworks, loud noises/gunshots and thunderstorms frequently co-occurred. Female dogs had higher odds of noise sensitivity compared to male dogs (OR = 1.3 $p < 0.001$), and neutered dogs had higher odds of being fearful of noises than intact dogs (OR = 1.73 $p < 0.001$). The dogs most fearful of noises also had higher odds of showing separation related behavior, being fearful in novel situations and required longer time to calm down after a stressful event compared to dogs less fearful of noises.

1. Introduction

Noise sensitivity is a common behavioral problem in dogs (Levine, 2009; Overall et al., 2001). One survey including 383 dog owners reported that almost half (49%, $n = 188$) of the owners stated that their dog was fearful of loud noises (Blackwell et al., 2005). In an online survey including more than 3500 dog owners, 2577 owners reported having a dog with noise sensitivity (Iimura, 2006). A survey including veterinary practitioners in Spain found that noise phobia was the fifth most frequent canine behavioral problem (Fatjó and Ruiz-de-la-Torre, 2006). Incidence data from behavior clinics may underestimate the underlying prevalence, since only a small number of dog owners are likely to seek specialist help. A study from New Zealand reported that only 15.8% of the owners with dogs that displayed a fearful response in situations with fireworks sought professional treatment (Dale et al., 2010). A Danish study found that owners of dogs with shooting phobia were less inclined to seek help with the behavior than owners of dogs with separation anxiety (Rugbjerg et al., 2003). Wells and Hepper (2000) reported that 68.3% of dogs purchased from an animal rescue shelter showed undesirable behavior within the first month and the majority, 53.4%, specified fearfulness as the major problem behavior. Behavioral responses of dogs with noise sensitivity can be extreme in nature and it may represent a serious welfare issue for the dog.

Dogs can be fearful of many different noises but the most commonly reported are fear of thunderstorms, fireworks, gunshots and engine noises (Levine, 2009; Sherman and Mills, 2008). A study found that fireworks was the most commonly reported noise aversion, followed by thunderstorms and gunshots (Iimura, 2006). Dogs with noise sensitivity may show a range of signs including restlessness/shaking/trembling, pacing, increased startle response, increased vigilance, hiding, panting, drooling, destructiveness, defecation, urination, vocalization, withdrawal, self-mutilation, loss of appetence, freezing, vomiting, expression of anal sacs, owner-seeking and yawning (Mills, 2005).

The fear response is a normal and self-protecting behavior, which can enable the dog to escape potential dangerous situations, but may, in some cases become inappropriate and negatively impact the dog's welfare. The terms anxiety, fear and phobia are often used interchangeably but they have different definitions.

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Anxiety describes the situation where the dog anticipates a negative outcome; it is an emotional response occurring prior to a stimulus/situation that the animal perceives as dangerous. In other words the dog shows signs of anxiety to a situation or stimuli which might occur, but the anxiety may be displayed in the absence of an identifiable stimulus as well (Landsberg et al., 2013). Fear is an adaptive response where a response starts when the animal perceives the presence of stimuli considered to potentially be dangerous. The fear response allows the dog to avoid dangerous situations and increase chances of survival. Phobia is a sudden, excessive and profound fear. The phobic symptoms persist after the stimuli are removed or have disappeared and the phobic reaction may occur in the absence of the trigger. Phobias are not an adaptive response and interfere with normal functioning (Palestrini, 2009).

Factors proposed to contribute in development and progression of noise aversions are genetic, traumatic experience associated with noise exposure, social transmission (learned from other fearful dogs), and owner responses (inappropriate owner response or responses reinforcing the dog's fear) (Landsberg et al., 2013). Breed has been reported as a risk factor for fear sensitivities and Blackwell et al. (2013) found that twelve breeds or breed types were less likely to show fear responses to noises than cross-breeds and their data suggest that some breeds may predisposed to fear of loud noises. Iimura (2006) found that noise sensitivity with an acute onset seem to be associated with a fear response; while noise sensitivity with less acute onset seem to be associated with an anxiety response. These factors also showed significant association with breed, the age the dog was obtained and the age at onset of the problem; Owners of hounds, toy breeds and mixed breeds more commonly reported an acute-onset history with fear-related problems, whereas owners of terriers, intact bitches and dogs rehomed around 1 month of age more typically reported a non-acute onset (Iimura, 2006; Sherman and Mills, 2008). Rugbjerg et al. (2003) reported a significant effect of owner types (the owner not being a dog breeder was found a risk factor), and that some breeds (Poodles, retrieving/flushing dogs, Sheepdogs, Spitz dogs and terriers) had a higher odds of shooting phobia. Vucinic et al. (2013) did not find any significant differences in the incidence of noise related fears among dogs of mixed or pure breeds, male or female or among intact or neutered dogs. A survey of 69 cases of thunderstorm phobia in dogs indicated that there may be a predisposition among some herding breeds (McCobb et al., 2001).

The primary aim of the study was to describe the distribution of noise sensitivity in different dog breeds. Furthermore the aim was to investigate the influence of sex, age, and neuter status and the co-morbidity of fear responses.

2. Materials and methods

An extensive web survey on noise sensitivity was conducted in collaboration with 17 breed clubs. The included breeds represent breeds where the breed clubs had participated in a voluntarily general health and behavior survey. Dog owners were encouraged to participate in the survey with information about their dogs, including dogs presently alive and dogs that were recently deceased.

The owners answered four items concerning reactions to loud noises including gunshots, fireworks, thunderstorms and heavy traffic. The questions were:

- Does your dog show signs of being fearful during loud noises/gunshot?
- Does your dog show signs of being fearful in situations with fireworks?
- Does your dog show signs of being fearful during thunderstorms?
- Does your dog show signs of being fearful in situations with heavy traffic?

The answers were in a scale from 1 to 5;

1—no signs,
2—mild signs,
3—moderate signs,
4—strong signs,
5—very strong signs.

Two new variables were created, both based on the score in all the four fear groups. A dog was classified as "fearful" if it had a score of minimum 4 in at least one of the 4 categories and was treated as a categorical variable (1 = fearful, 0 = not fearful). The other variable "sumfear" was a simple sum of the scores in the four categories and was treated as a continuous variable.

In addition, the following fear responses were included in this study based on the questions; "Does your dog show signs of separation related behavior when left alone?" (1—no signs, 2—mild signs, 3—moderate signs, 4—strong signs and 5—very strong signs), "Does your dog show signs of being fearful in novel situations?" (1—no signs, 2—mild signs, 3—moderate signs, 4—strong signs and 5—very strong signs), and "Does your dog quickly calm down after being in a stressful situation?" (1—very quickly, 2—quickly, 3—neither quickly nor slowly, 4—slowly and 5—very slowly).

A total of 5257 dogs were included in the study distributed over 17 breeds. The number from each breed is shown in Table 1.

2.1. Description of the studied population

The mean age of all the dogs was 4.93 years (4900 had information about age), if the owner has given information about a deceased dog, the age given is the age of the dog when it died.

The sex distribution of the total material was 54.8% (n = 2876) females and 45.2% (n = 2372) males. Nine owners did not report sex. In total 13.43% (n = 693) of the dogs were neutered, the neuter frequency between the sexes is almost the same 13.57% (382) of the females and 13.28% (311) of the males are neutered.

2.2. Statistical analysis

All statistical analyses were performed using Stata version 12.0.

An analysis of variance (ANOVA) was performed to explore potential effects of breed in the four different categories followed by a Tukey post hoc test. An ordinal logistic regression model was performed to measure the association between noise sensitivity, breed, sex and age. Noise sensitivity was the dependent variable and breed, sex and age were independent variables. Co-occurrence between the categories of noise aversions was measured with Spearman's correlation test, odds ratios (OR) were utilized for measurement of co-occurrence between noise aversions and fear responses in other situations.

3. Results

3.1. Noise sensitivity

Most fearful dogs reacted to fireworks and gunshots, while the reaction to heavy traffic and thunderstorms was less pronounced. 21.17% (1076 of 5082) showed strong or very strong signs of being fearful during fireworks, 14.4% (740 of 5139) showed strong or very strong signs of being fearful in situations with loud noises/gun shots, 10.14% (520 of 5128) showed strong or very strong signs of being fearful during thunderstorms and 2.85%, (148 of 5175)

Table 1
Breed distribution, sorted by frequency.

Breed	Frequency	Percent	Females (%)	Males (%)
Boxer	810	15.41	453 (56.2)	353 (43.8)
Collie	561	10.67	319 (56.9)	242 (43.1)
Irish Soft Coated Wheaten Terrier	404	7.68	219 (54.2)	185 (45.8)
Nova Scotia Duck Tolling Retriever	403	7.67	220 (54.7)	182 (45.3)
Norwegian Buhund	359	6.83	180 (50.3)	178 (49.7)
Lagotto Romagnolo	332	6.32	166 (50.2)	165 (49.8)
Shiba Inu	319	6.07	168 (52.7)	151 (47.3)
Miniature Schnauzer	318	6.04	177 (55.8)	140 (44.2)
Great Dane	278	5.29	152 (54.7)	126 (45.3)
Welsh Corgi	274	5.21	161 (58.8)	113 (41.2)
Cairn Terrier	255	4.85	136 (53.3)	119 (46.7)
Pointer	255	4.85	142 (55.7)	113 (44.3)
Bullmastiff	237	4.51	132 (55.7)	105 (44.3)
Chinese Crested	172	3.27	92 (53.8)	79 (46.2)
Giant Schnauzer (Riesenschnauzer)	162	3.08	96 (59.3)	66 (40.7)
Standard Schnauzer	86	1.63	42 (48.8)	44 (51.2)
Bouvier des Flandres	32	0.60	21 (65.6)	11 (34.4)
Total	5257	100	2876 (54.8)	2372(45.2)

showed strong or very strong signs of being fearful in situations with heavy traffic. In total, 10.51% (547 dogs) showed strong signs and 12.68% (660 dogs) showed very strong signs of fear across all the four categories (Table 2).

There were distinct differences regarding noise aversions between the breeds (Tables 3–6).

The frequency of fear in situations with fireworks (category 4/5) varied greatly between breeds from 8.8% in Chinese Crested to 32% in Norwegian Buhund (Table 3). The difference between breeds was statistically significant ($F = 18.09$, $p < 0.001$). A Tukey post hoc test revealed that the difference between the breeds with the highest frequency of fearful dogs in situations with fireworks (Norwegian Buhund, Shiba Inu, Irish Soft Coated Wheaten Terrier, Lagotto Romagnolo, Cairn Terrier and Collie) was significantly different compared to Boxer, Chinese Crested, Minature Schnauzer, Great Dane, Giant Schnauzer and Pointer ($p < 0.05$) (Table 3).

The frequency of loud noises/gunshots also showed significant difference between the breeds ($F = 18.80$, $p < 0.001$) (Table 4). Lagotto Romagnolo showed highest frequencies of strong and very strong signs (24%) of fear in situations with loud noises/gunshots. Pointer had lowest frequency of fearful dogs (4%). Pairwise comparison revealed that fear of loud noises/gunshots was statistically significantly different in breeds with high frequency of fearful dogs (Lagotto Romagnolo, Irish Soft Coated Wheaten Terrier, Norwegian Buhund, Shiba Inu, Nova Scotia Duck Tolling Retriever and Collie) (Table 4) compared to Great Dane, Boxer and Pointer ($p < 0.05$).

A separate comparison was done between hunting breeds (Pointer and Nova Scotia Duck Tolling Retriever) and the other breeds with regards to gunshots, and the odds of being fearful of gunshots was 1.4 times higher in the 15 breeds compared to the hunting breeds (95% CI 1.1–1.8, Chi-square 6.12 $p = 0.01$). Fig. 1 shows frequency in percent between the groups.

Table 2
The distribution of "fearfulness" scores across all 17 breeds sorted according to highest score (1–5) from the four categories combined.

Category	Frequency	Percent
1—No signs	1917	36.84
2—Mild signs	1298	24.95
3—Moderate signs	781	15.01
4—Strong signs	547	10.51
5—Very strong signs	660	12.68
Total	5203	100

54 missing observations.

There was also a statistically significant difference of fearfulness between the breeds ($F = 16.79$, $p < 0.001$) during thunderstorms (Table 5). Norwegian Buhund showed the highest frequency of strong and very strong signs of fear during thunderstorms. Bouvier des Flandres and Great Dane had the lowest frequency of fearful dogs. A Tukey post hoc test revealed that fear of thunder was statistically significantly different in breeds with high frequency of fearful dogs (Norwegian Buhund, Irish Soft Coated Wheaten Terrier, Lagotto Romagnolo and Nova Scotia Duck Tolling Retriever) (Table 5) compared to Boxer, Chinese Crested, Great Dane and Bouvier des Flandres ($p < 0.05$).

Fear of loud noises from heavy traffic was less pronounced compared to the other fear categories. However, for fear in situations with heavy traffic, there was a statistically significant difference between the breeds ($F = 8.37$, $p < 0.001$) (Table 6). Nova Scotia Duck Tolling Retriever showed the highest frequencies of strong and very strong signs of fear in situations with heavy traffic. Bouvier des Flandres and Great Dane had the lowest frequency of fearful dogs. Also for fear of thunder the breeds with highest frequency of fearful dogs (Nova Scotia Duck Tolling Retriever and Norwegian Buhund) (Table 6) were significantly different compared to Boxer, Great Dane and Bouvier des Flandres ($p < 0.05$).

Table 7

Fig. 1. Proportion of dogs fearful of gunshots in hunting breeds and non-hunting breeds, "Does your dog show signs of being fearful during loud noises/gunshot?" in the scale 1–5 (1—no signs, 2—mild signs, 3—moderate signs, 4—strong signs, 5—very strong signs).

Table 3
Distribution of "Fearfulness in situations with fireworks" sorted from high to low (percent of fearful dogs) ($n = 5082$).

Breed	1	2	3	4	5	Total
Norwegian Buhund	35.4 *125*	21.3 *75*	11.3 *40*	9.9 *35*	22.1 *78*	(%) *353 (n)*
Shiba Inu	43.9 *136*	13.9 *43*	10.7 *33*	11.9 *37*	19.7 *61*	*310*
Irish Soft Coated Wheaten Terrier	43.0 *159*	14.6 *54*	11.1 *41*	12.2 *45*	19.0 *70*	*369*
Lagotto Romagnolo	36.0 *117*	20.9 *68*	12.9 *42*	16.9 *55*	13.2 *43*	*325*
Cairn Terrier	35.5 *89*	19.1 *48*	15.5 *39*	11.6 *29*	18.3 *46*	*251*
Collie	43.8 *237*	18.7 *101*	14.2 *77*	9.6 *52*	13.7 *74*	*541*
Nova Scotia Duck Tolling Retriever	43.7 *169*	19.1 *74*	14.0 *54*	9.0 *35*	14.2 *55*	*387*
Standard Schnauzer	47.1 *40*	20.0 *17*	10.6 *9*	5.9 *5*	16.5 *14*	*85*
Welsh Corgi	39.6 *106*	24.3 *65*	14.9 *40*	9.0 *24*	12.3 *33*	*268*
Miniature Schnauzer	52.6 *165*	18.8 *59*	9.6 *30*	9.6 *30*	9.6 *30*	*314*
Bullmastiff	54.0 *128*	20.7 *49*	7.2 *17*	11 *26*	7.2 *17*	*237*
Bouvier des Flandres	59.4 *19*	15.6 *5*	9.4 *3*	12.5 *4*	3.1 *1*	*32*
Giant Schnauzer (Riesenschnauzer)	47.5 *76*	28.1 *45*	9.4 *15*	7.5 *12*	7.5 *12*	*160*
Pointer	60.2 *151*	17.9 *45*	7.2 *18*	8.8 *22*	6.0 *15*	*251*
Great Dane	55.7 *147*	19.7 *52*	14.0 *37*	5.7 *15*	4.9 *13*	*264*
Boxer	65.5 *509*	16.7 *130*	8.2 *64*	5.0 *39*	4.5 *35*	*777*
Chinese Crested	60.1 *95*	19.6 *31*	11.4 *18*	2.5 *4*	6.3 *10*	*158*
Total	48.56 2468	18.91 961	11.35 577	9.23 469	11.94 607	100.00 5082

3.2. Correlation between different types of noise sensitivity

There was a positive correlation between the different types of fear, varying from 0.29 to 0.78 (Table 8). The strongest positive correlation was between fear of fireworks and fear of gunshots/loud noises, ($r_s = 0.78$, $p < 0.001$). The other correlation coefficients and statistical significance can be found in Table 8

Of the 1076 dogs where the owner scored their dog at 4 or 5 on the scale of being fearful during fireworks, 44.7% (481) of the dogs also showed strong or very strong signs of fear during thunderstorms, while only 14.9% (160) of the owners with fearful dogs during fireworks answered that their dog was not afraid at all during thunderstorms. Dogs fearful during fireworks also showed a high frequency of being fearful in situations with loud noises/gunshots; 61.7% (664) showed strong or very strong signs of being fearful during fireworks and in situations with loud noises/gunshots, only 7.6% (82) of the dogs were not fearful in situations with gunshots, but fearful during fireworks. The odds of being fearful of gunshots in dogs also afraid of fireworks was 1242 times higher than in dogs unafraid of fireworks (95% CI 700–2204, Chi-square 2421 $p < 0.001$).

Breed, sex and age were found to have a significant effect on "fearfulness" in situations with loud noises; chi-square 97.71, $p < 0.001$, breed ($p < 0.001$, 95% CI 1.03–1.05), sex ($p < 0.001$, 95% CI 0.73–0.89) and age ($p < 0.001$, 95% CI 1.02–1.05, OR 1.034). For age there was a positive correlation; for each additional year of age there was a 3.4% increase in the odds of the dog showing strong or very strong signs of fear when exposed to loud noise (Fig. 2).

3.3. Sex and neuter status

The studied population consists of slightly more females than males, 54.8% females and 45.2% males.

The frequency of neutering did not differ significantly between female and male dogs in the study.

Table 4
Distribution of "Fearfulness in situations with loud noises/gunshots?" sorted from high to low (percent of fearful dogs) ($n = 5139$).

Breed	1	2	3	4	5	Total
Lagotto Romagnolo	36.7	26.7	12.7	15.8	8.2	(%)
	121	*88*	*42*	*52*	*27*	*330* (*n*)
Irish Soft Coated Wheaten Terrier	38.7	23.8	14.3	13.5	9.7	
	143	*88*	*53*	*50*	*36*	*370*
Norwegian Buhund	44.8	21.7	11.0	10.1	12.4	
	159	*77*	*39*	*36*	*44*	*355*
Shiba Inu	50.3	19.4	11.5	9.6	9.2	
	158	*61*	*36*	*30*	*29*	*314*
Nova Scotia Duck Tolling Retriever	43.0	27.0	11.8	9.3	9.0	
	172	*108*	*47*	*37*	*36*	*400*
Collie	48.4	19.0	15.2	8.2	9.3	
	265	*104*	*83*	*45*	*51*	*548*
Welsh Corgi	43.1	25.3	14.5	10.0	7.0	
	116	*68*	*39*	*27*	*19*	*269*
Cairn Terrier	41.1	25.7	17.4	9.5	6.3	
	104	*65*	*44*	*24*	*16*	*253*
Miniature Schnauzer	53.2	21.3	11.9	8.4	5.2	
	165	*66*	*37*	*26*	*16*	*310*
Bouvier des Flandres	65.6	18.8	3.1	9.4	3.1	
	21	*6*	*1*	*3*	*1*	*32*
Bullmastiff	52.8	23.8	12.3	5.5	5.5	
	124	*56*	*29*	*13*	*13*	*235*
Standard Schnauzer	56.5	18.8	14.1	5.9	4.7	
	48	*16*	*12*	*5*	*4*	*85*
Giant Schnauzer (Riesenschnauzer)	58.2	22.8	10.1	5.0	3.8	
	92	*36*	*16*	*8*	*6*	*158*
Chinese Crested	53.3	28.4	10.0	6.5	1.8	
	90	*48*	*17*	*11*	*3*	*169*
Great Dane	56.9	24.2	12.7	4.2	1.9	
	148	*63*	*33*	*11*	*5*	*260*
Boxer	63.2	22.0	9.0	3.8	2.0	
	505	*176*	*72*	*30*	*16*	*799*
Pointer	87.3	6.4	2.4	1.2	2.8	
	220	*16*	*6*	*3*	*7*	*252*
Total	51.59	22.22	11.79	8.00	6.40	100.00
	2651	1142	606	411	329	5139

The frequency of "fearfulness" was slightly higher in female dogs than in male dogs ($n = 5189$), 24.9% of female dogs, and 21.2% of male dogs were fearful of noises (score 4 or 5). In addition, for all of the separate categories of noise sensitivity a significant effect of both sex and neutering was observed. Females had 1.3 times higher odds than males (95% Confidence interval 1.159–1.551, Chi-square 15.62, $p < 0.001$). There was a positive association between fearfulness and neutering. The odds of neutering were 1.72 higher

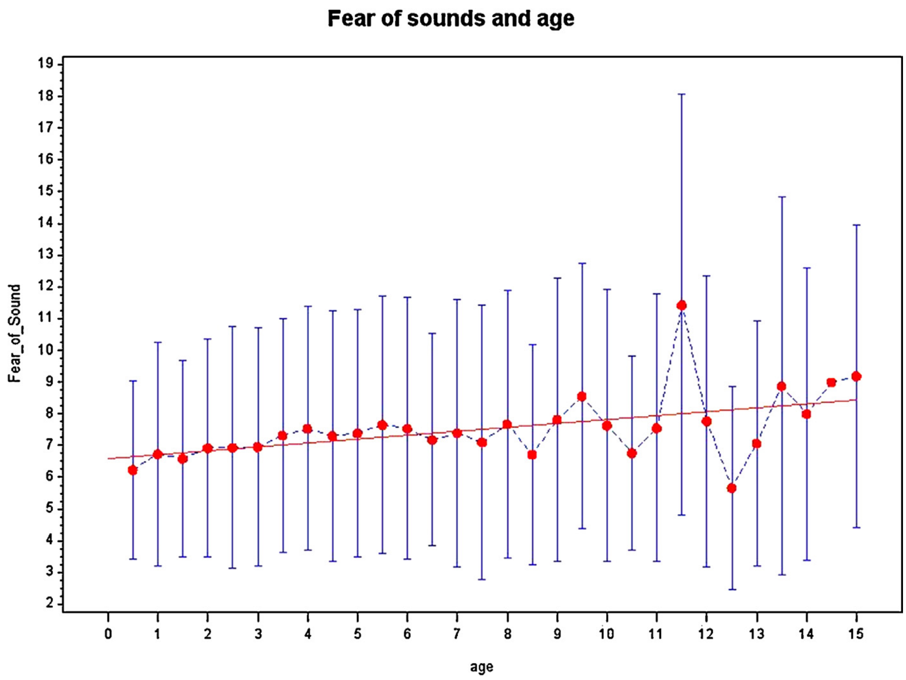

Fig. 2. Correlation between fear of sound (average of the variable "soundfear") and age in years, dots are summary values with corresponding measures of dispersion.

Table 5
Distribution of "Fearful during thunderstorms", sorted from high to low (percent of fearful dogs) ($n = 5128$).

Breed	1	2	3	4	5	Total
Norwegian Buhund	51.3	18.3	10.1	8.7	11.6	(%)
	182	*65*	*36*	*31*	*41*	*355* (*n*)
Irish Soft Coated Wheaten Terrier	56.5	16.0	10.9	7.9	8.7	
	208	*59*	*40*	*29*	*32*	*368*
Lagotto Romagnolo	52.0	22.6	11.2	9.9	4.3	
	168	*73*	*36*	*32*	*14*	*323*
Nova Scotia Duck Tolling Retriever	52.5	26.0	7.3	7.0	7.0	
	208	*103*	*29*	*28*	*28*	*396*
Standard Schnauzer	61.6	20.9	3.5	10.5	3.5	
	53	*18*	*3*	*9*	*3*	*86*
Cairn Terrier	54.8	18.3	13.9	7.5	5.6	
	138	*46*	*35*	*19*	*14*	*252*
Collie	56.9	20.2	10.5	5.9	6.5	
	308	*109*	*57*	*32*	*35*	*541*
Welsh Corgi	50.6	23.3	14.0	6.6	5.5	
	137	*63*	*38*	*18*	*15*	*271*
Shiba Inu	61.0	16.6	10.2	6.0	6.0	
	191	*52*	*32*	*19*	*19*	*313*
Pointer	68.3	17.9	5.2	5.2	3.6	
	172	*45*	*13*	*13*	*9*	*252*
Bullmastiff	70.6	15.3	7.2	4.3	2.6	
	166	*36*	*17*	*10*	*6*	*235*
Giant Schnauzer (Riesenschnauzer)	71.9	15	6.9	3.1	3.1	
	115	*24*	*11*	*5*	*5*	*160*
Miniature Schnauzer	70.5	14.7	9.6	1.9	3.2	
	220	*46*	*30*	*6*	*10*	*312*
Boxer	77.1	14.3	5.3	2.6	0.6	
	613	*114*	*42*	*21*	*5*	*795*
Chinese Crested	74.1	17.7	5.3	1.2	1.8	
	126	*30*	*9*	*2*	*3*	*170*
Great Dane	72.3	16.1	9.0	2.3	0.4	
	193	*43*	*24*	*6*	*1*	*267*
Bouvier des Flandres	93.8	0	6.3	0	0	
	30	*0*	*2*	*0*	*0*	*32*
Total	62.95	18.06	8.85	5.46	4.68	100.00
	3228	926	454	280	240	5128

in fearful dogs than in non-fearful dogs (95% CI 1.405–2.109, Chi-square 27.83, $p < 0.001$).

3.4. *Correlation of noise sensitivity and fear responses in other situations*

There were higher odds (OR = 3) of separation related fear in dogs being fearful to loud noises (95% CI 2.100–4.540, Chi-square 36.05, $p < 0.001$). This was also true for all separate categories of noise sensitivities; the odds of separation related fear was 7.7 times higher in dogs fearful to heavy traffic (95% CI 4.677–12.621). For both fear in thunderstorms (95% CI 2.021–4.456, Chi-square 32.97, $p < 0.001$) and fear of gunshots (95% CI 2.141–4.403, Chi-square 40.72, $p < 0.001$) the odds of separation related fear was higher (OR = 3). The odds of separation related fear was 2.2 times higher in dogs fearful of fireworks (95% CI 1.561–3.123, Chi-square 20.12, $p < 0.001$).

Dogs with noise sensitivity more often showed signs of being fearful in novel situations compared to dogs resistant to noise (OR 18.3, 95% CI 11.9–28.3, Chi-square 287,9, $p < 0.001$). Dogs with noise sensitivity also took a longer time to calm down after stressful situations than dogs with no noise aversions (OR 3.6, 95% CI 1.578–8.096, Chi-square 10.6, $p = 0.001$).

4. Discussion

This study describes high frequencies of noise sensitivity in dog breeds and significant effects of breed, sex and age. Using web-based questionnaires we were able to get a high number of responses describing dogs from several breeds. Surveys based on questionnaires may of course be sensitive to owners interpretation, but studies have shown that questionnaires can serve as an accurate and reliable tool for assessing fearful behavior in dogs (Tiira and Lohi, 2014). Hsu and Serpell (2003) have also validated that a questionnaire may be a reliable and valid method of assessing behavior and temperament traits in dogs.

In average almost 23% of the owners reported their dog to be fearful of noises. Similar numbers were found in a study from UK (Blackwell et al., 2013), but other studies have reported higher numbers, Iimura (2006) found from a large online survey that out of over 3500 dog owners, 2577 owners reported having a noise-aversive dog. In a smaller subset in the study of Blackwell et al. (2013) almost half of the responding owners reported that their dog showed at least one behavioral sign typical to fear when exposed to noises, and in a smaller study from Belgrade, 40.14% of the owners reported noise-related fears in their dogs (Vucinic et al., 2013). Owner based questionnaires may underestimate the actual prevalence since an unexperienced owner may miss signs of fear in the dog. It is also difficult to know if some owners and breeders may underreport some of these traits. Even though there is some variation in estimates from different studies, there is a general agreement that the prevalence of fear related to loud noises is a very common problem in dogs.

This study only included the owner's general opinion if the dog showed fear in four situations; fireworks, gunshots, heavy traffic and thunderstorms (scored 1–5) and did not include the specific behavioral sign the dog showed (e.g. barking, hiding, shaking, pacing, elimination). As expected fireworks was the most common sound causing the dog to be fearful, followed by gunshots, thunderstorms and heavy traffic. This may be explained by the fact that fireworks usually are particular loud and long lasting and also an unusual sound for the dog.

Table 6
Distribution of "Fear in situations with heavy traffic?" sorted from high to low (percent of fearful dogs) ($n = 5175$).

Breed	1	2	3	4	5	Total
Nova Scotia Duck Tolling Retriever	65.0	20.1	8.7	5.5	0.7	(%)
	262	*81*	*35*	*22*	*3*	*403* (*n*)
Norwegian Buhund	60.6	19.8	14	3.9	1.6	
	217	71	50	14	6	358
Irish Soft Coated Wheaten Terrier	69.8	18.0	7.8	3.0	1.4	
	258	*67*	*29*	*11*	*5*	*370*
Chinese Crested	67.3	17.0	12.3	2.9	0.6	
	115	*29*	*21*	*5*	*1*	*171*
Welsh Corgi	77.9	12.9	5.9	2.6	0.7	
	212	*35*	*16*	*7*	*2*	*272*
Giant Schnauzer (Riesenschnauzer)	80.5	13.2	3.1	2.5	0.6	
	128	*21*	*5*	*4*	*1*	*159*
Shiba Inu	71.9	18.3	6.6	2.5	0.6	
	228	*58*	*21*	*8*	*2*	*317*
Pointer	80.3	14.2	2.4	1.6	1.6	
	204	*36*	*6*	*4*	*4*	*254*
Collie	78.0	14.2	4.9	2.0	0.9	
	428	*78*	*27*	*11*	*5*	*549*
Lagotto Romagnolo	64.3	24.7	9.2	1.2	0.6	
	211	*81*	*30*	*4*	*2*	*328*
Bullmastiff	79.8	13.9	4.6	1.3	0.4	
	189	*33*	*11*	*3*	*1*	*237*
Miniature Schnauzer	76.3	17.1	5.1	1.6	0	
	241	*54*	*16*	*5*	*0*	*316*
Boxer	79.3	14.1	5.1	0.9	0.6	
	634	*113*	*41*	*7*	*5*	*800*
Cairn Terrier	75.1	15.8	7.9	1.2	0.0	
	190	*40*	*20*	*3*	*0*	*253*
Standard Schnauzer	75.6	15.1	8.1	1.2	0	
	65	*13*	*7*	*1*	*0*	*86*
Great Dane	85.2	10.4	3.7	0.7	0	
	230	*28*	*10*	*2*	*0*	*270*
Bouvier des Flandres	78.1	18.8	3.1	0	0	
	25	*6*	*1*	*0*	*0*	*32*
Total	74.14	16.31	6.69	2.14	0.71	100.00
	3837	844	346	111	37	5175

Table 7
Summary of the breeds with the highest (top four) and lowest (bottom four) frequencies of fearful dogs in the four categories of noise sensitivity.

	Fireworks	Gunshots	Thunderstorms	Heavy traffic
High frequency	Norwegian Buhund	Lagotto Romagnolo	Norwegian Buhund	NSDTR
	Shiba Inu	ISCWT	ISCWT	Norwegian Buhund
	ISCWT	Norwegian Buhund	Lagotto Romagnolo	ISCWT
	Lagotto Romagnolo	Shiba Inu	NSDTR	Chinese Crested
Low frequency	Pointer	Chinese Crested	Boxer	Cairn Terrier
	Great Dane	Great Dane	Chinese Crested	Standard Schnauzer
	Boxer	Boxer	Great Dane	Great Dane
	Chinese Crested	Pointer	Bouvier des Fl.	Bouvier des Fl.

NSDTR—Nova Scotia Duck Tolling Retriever, ISCWT—Irish Soft Coated Wheaten Terrier and Bouvier des Fl. —Bouvier des Flandres.

The 17 breeds included in this study represented different breed groups and we believe that the large number of dogs included provides a good estimate of noise sensitivity in dogs. The frequencies of dogs from each breed differ from 32 (Bouvier des Flandres) to 810 (Boxer). The study showed significant differences between the breeds regarding noise sensitivity; Norwegian Buhund, Irish Soft Coated Wheaten terrier and Lagotto Romagnolo were the breeds with the highest prevalence of fear across the following three categories: fireworks, gunshots and thunderstorms. Breeds that showed consistently low frequencies of noise sensitivity were Boxer, Chinese Crested and Great Dane. In all the four categories of noise sensitivity (fireworks, gunshots, thunderstorms and heavy traffic) there were statistically significant difference between the breeds with the highest prevalence compared to breeds with lower frequencies of fearful dogs in the respective categories (Tables 3–7). One study indicated that there may be a breed predisposition for thunderstorm phobia in herding dogs (McCobb et al., 2001), but we are not aware of any general documentation indicating that herding dogs are more noise-sensitive than other breeds. In this study only one (Norwegian Buhund) of three herding breeds (the other two being Collie and Bouvier des Flandres) showed high prevalence of noise aversions. Interestingly, owners of Pointer dogs reported

Table 8
Spearman correlation coefficient and statistical significance between the four categories of fear of noises in different situations, r_s (p), $n = 4967$.

	Heavy traffic	Thunderstorms	Gunshots/loud noises	Fireworks
Heavy traffic	–	0.34 (<0.001)	0.36 (<0.001)	0.29 (<0.001)
Thunderstorms		–	0.70 (<0.001)	0.73 (<0.001)
Gunshots/loud noises			–	0.78 (<0.001)

that 87.3% of the dogs were not fearful in situations with gunshots. As the Pointer is bred for hunting, there may have been a selection for tolerance to gunshots/loud noises. This study showed that the included breeds not used for hunting had higher odds of being fearful of gunshots than hunting breeds (Pointer and Nova Scotia Duck Tolling Retriever). This is similar to findings from Blackwell et al. (2013); where popular gundog breeds were less likely to show fear responses to noises (compared to cross-breeds). The significant difference between the breeds in all the categories of noise sensitivity indicates that risk alleles of anxiety genes may have been accumulated in specific breeds during selection as a random effect (popular sires, line breeding, genetic drift) or as a correlated effect to other desired traits.

Dogs fearful of fireworks also showed a higher frequency of being afraid of thunderstorms and gunshots. There was a positive correlation between showing signs of fear in all four types of recorded loud noises. The strongest correlation was between fireworks and gunshots, but a strong correlation was also found between fireworks-thunderstorms and thunderstorms-gunshots, while a weaker correlation was found between heavy traffic and the other categories. This could suggest that fearful dogs may commonly generalize fear of one loud sound to another, especially with regards to particular loud/explosive sounds. The dog's underlying temperament might also be an explanation, as a dog that is anxious in general naturally will react to loud noises no matter the origin of the sound. Noise from traffic is less intense and more common in most dogs' everyday life. This give dogs a better chance to adapt to the noise that may explain the weaker correlation.

The frequency of noise sensitivity (combined for all four categories) was higher in female dogs (OR = 1.3). This is in contrast to other studies where sex of the dog was not found to be a risk factor (McCobb et al., 2001; Vucinic et al., 2013) (this may be due to the low number of dogs included in these two studies) while Blackwell et al. (2013) found that males had a significant higher fear of gunshots than females (irrespective of neuter status).

Neutered dogs also have higher odds of being noise sensitive. This may suggest that hormonal factors play a role in development of noise aversions, but this needs to be investigated further, and may be an example of a negative influence of neutering on behavior traits. Another likely explanation may be that some owners believe that neutering may help to reduce the problem. In this study the owners were not asked about the reason for neutering.

Each additional year of age is associated with a 3.4% increase in the odds of reporting the dog of being fearful. Behavioral problems can often lead to relinquishment of the dog and in some cases euthanasia, which might have resulted in a lower problem in the (remaining) population of older dogs. One explanation for the increasing trend with age could be that fear sensitivity is not considered to be serious enough for the owner to seek treatment or start systematic training, and/or that there is in general, low effect of treatment. Dale et al. (2010) found that only 15.8% of the owners with dogs that displayed a fearful response in situations with fireworks sought professional treatment. This increase in fear with older age is interesting and indicates that dogs do not tend to handle fear better when they grow older.

Conflicting reports have been published on co-occurrence of noise sensitivity and separation related behavior. Overall et al. (2001) found a common co-occurrence of separation anxiety and noise phobia and thunderstorm phobia, while Blackwell et al. (2013) reported a low concordance between fearfulness of noises and other behaviors associated with fear or anxiety. In this study we found a significant relationship between noise sensitivity and separation related behavior. The odds of a dog showing separation related fear was three times higher in dogs that also were fearful of noises compared to dogs not fearful of noises. Interestingly the correlation was highest between separation related behavior and fear of noises from heavy traffic, and the weakest correlation was between separation related behavior and fear of fireworks. Symptoms of separation related behavior are nonspecific and include; destruction, vocalization, disarrangement of objects in the household (excessive motor activity), inappropriate urination or defecation and excessive salivation. In this study the owners are asked if the dog shows any signs of showing separation related fear when left alone, and the limitations of this should be considered as the owners only record their dog's severity (in the scale 1–5) of fear and not the specific signs. Dogs with noise sensitivity also showed high co-occurrence with being fearful of novel situations. This might also be explained by the dogs' underlying temperament and that some dogs are more fearful in general. The ability to quickly recover from stressful events give an indication of the dog's nerve stability and hardness (lack of a lasting effect of a pleasant or frightening experience) (Wilsson, 1997). Fearful dogs in this study had a longer recovery time after a stressful event than dogs not being fearful, suggesting that an overall general nervousness or anxious temperament may be underlying. These findings suggest that there may be an underlying predisposition influencing both noise sensitivity and fear in other situations.

5. Conclusion

The study showed that approximately 23% of the owners reported their dogs to be fearful of loud noises and that there were significant differences between breeds. Certain breeds had consistently higher frequencies of fear compared to others. This indicates a strong genetic influence on fearfulness to loud noises and an accumulation of risk alleles in some breeds. Further studies should be performed aiming at the identification of risk loci, which in the future may be used as a tool in breeding programs aiming at the reduction of the frequency of these disorders.

Conflict of interest statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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A GENOME-WIDE ASSOCIATION STUDY FOR NOISE SENSITIVITY IN 5 DOG BREEDS

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Abstract

Noise sensitivity is a common problem in dogs and show significant differences in frequency between breeds. In this study we searched for genetic factors that could be associated with fearfulness of loud noises. A genome-wide association study (GWAS) of over 400 dogs across five breeds (Collie, Irish softcoated wheaten terrier, Nova Scotia duck tolling retriever, Norwegian Buhund and Staffordshire bull terrier) did not reveal significant genome-wide associations. The study may however be underpowered due to potential genetic heterogeneity within and between breeds. Allele frequencies between breeds were evaluated for the top SNPs from each GWA analysis and in an area around an anxiety candidate gene *DRD2,* and were found to vary greatly both within and between breeds, in addition many markers show nearly a total fixation. The allele frequencies of the major allele also showed opposite direction in cases and controls in some markers between breeds.

Introduction

Noise sensitivity is a common problem in dogs (Blackwell, Bradshaw, & Casey, 2013; Overall, Dunham, & Frank, 2001; Storengen & Lingaas, 2015) and include a wide array of emotional states, from showing subtle symptoms of discomfort to phobic reactions. Dogs are most commonly fearful of noises from fireworks, thunderstorms, gunshots and engines (Levine, 2009; Sherman & Mills, 2008). Having a dog with noise sensitivity potentially creates welfare issues for the dog and could inflict limitations to the owner's everyday life.

Anxiety and fear are normal emotional responses to threatening situations, but in anxiety disorders like noise phobia in dogs and panic disorder in humans, the responses are profound, exaggerated and/or prolonged (Sokolowska & Hovatta, 2013). Anxiety disorders in humans were the most common mental disorders within the EU in 2010 with a prevalence of 14 % (Wittchen et al., 2011) and heritability estimates about 30-50% (Shimada-Sugimoto, Otowa, & Hettema, 2015). In spite of the high heritability of anxiety-traits, identification of genes associated with behavior have been difficult and the progress have been slow compared to identification of genes involved in monogenic diseases. Behavioral disorders have a complex etiology and are probably influenced by several environmental and genetic components. Researchers studying noise phobia in Border collies, Australian shepherds, Bearded collies, Belgian shepherds, Belgian tervurens, Great danes and German shepherds found moderate association in regions on chromosome 5, 8 and 10 but none reached genome-wide significance in a genome-wide association analysis (Yokoyama, n.d.). Genome-wide association studies are promising as an approach for the study of complex diseases and traits in general, and have identified a large number of loci involved in human complex traits, however the result of these studies can explain only a small proportion of trait heritability (Eichler et al., 2010). This "missing heritability" has shifted the focus from the hypothesis that common variants cause common diseases, towards rare variants exerting larger effect sizes (McClellan, Susser, & King, 2007). Human genome-wide association studies aiming to identify common variants of the anxiety related personality trait neuroticism and panic disorder, have supported the involvement of a relatively large number of small effect size common and rare variants in the predisposition to anxiety disorders (Calboli et al., 2010;

Otowa et al., 2009; Sciortino et al., 2013). However, robust associations in human studies of complex traits with a high level of genetic heterogeneity depends greatly on the sample size; e.g. GWAS studies of schizophrenia including nearly 40,000 cases and over 100,00 controls found 108 independent associated loci (Ripke et al., 2014). In dog studies, to date, only one genome-wide association analysis study has achieved genome-wide statistical significance identifying a gene related to a canine behavioral disorder, linking Cadherin 2 (*CDH2*) to compulsive disorder susceptibility in Doberman pinchers (Dodman et al., 2010). However, other mapping strategies have found genes linked to behavior in dogs. A study of Labrador retrievers being trained as guide dogs found that polymorphisms in *COMT* (catechol-O-methyltransferase enzyme) and *SLC1A2* (glutamate transporter) were significantly related to activity level (Takeuchi, Hashizume, et al., 2009a). Takeuchi et al. also found that a polymorphism in *SLC1A2* was significantly related to aggression to strangers in the Shiba inu breed (Takeuchi, Kaneko, et al., 2009b). Candidate gene studies examining human-directed aggression in English cocker spaniels have identified risk and protective haplotypes in the dopamine receptor D1 (*DRD1*), serotonin receptors 1D, 2C (*HTR1D* and *HTR2C*) and neurotransmitter transporter *SLC6A1* (Våge et al., 2010) (Vage et al., 2008). Markers in the D4 dopamine receptor gene (*DRD4*) were found to be associated with fearfulness and fearlessness in a study of Korean native dogs (Lee et al., 2008). The dopaminergic and serotonergic systems have also been studied in relation to behavioral traits in e.g. Golden retrievers and Belgian Malinois (Lit, Belanger, Boehm, Lybarger, & Oberbauer, 2013a; Lit et al., 2013b; van den Berg, Kwant, Hestand, van Oost, & Leegwater, 2005).

The canine genome provides unique opportunities in genetic research. Because of the formation of dog breeds, followed by strong selection, inbreeding, use of popular sires and genetic drift, there is large genetic variation between the breeds and small variation within breeds (Lindblad-Toh et al., 2005). Some breeds have long stretches of linkage disequilibrium (LD), up to 100 times more extensive than in humans (Sutter, 2004). The low within-breed genetic heterogeneity and the long LD make the dog particular suitable for studies of complex diseases. Improved understanding of canine behavioral genetics has not only the potential to benefit the dogs themselves but could also provide useful models for several human psychiatric disorders (van Rooy, Arnott, Early, McGreevy, & Wade, 2014).

The aim of this study was to identify regions/loci associated with noise sensitivity and to evaluate allele frequencies of the top SNPs from the analyses and in an area around a candidate gene (*DRD2*) within and between breeds.

Materials and methods

Animals

Privately-owned anxious and not anxious control dogs from 5 dog breeds (Collie, Irish softcoated wheaten terrier (ISWT), Nova Scotia duck tolling retriever (NSDTR), Norwegian Buhund (NB) and Staffordshire bull terrier (SB)) (N=420) were included in the study, with owner's consent (Table 1). The breeds represent variation in size and characteristics (herding dog, hunting/guard dog, working dog and companion dog).

[Table 1]

Sample collection and phenotyping

Blood samples were collected by certified veterinarians, in agreement with the provisions enforced by the Norwegian Animal Research Authority, in EDTA tubes and genomic DNA was extracted from the EDTA blood samples using E.Z.N.A blood DNA kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's recommendations, and subsequently stored at -20 degrees Celsius. The samples were collected (according to rules for ethical approval for collecting blood samples (FOR-2010-07-08-1085, FOR-1996-01-15-23, Regulation on Animal Experimentation). The phenotypic classification was done by one veterinarian based on the dog owner's response to an extensive questionnaire.

Genotyping and quality control

Genomic DNA was genotyped for approximately 170,000 SNPs using the Illumina 170k CanineHD Bead chip and the raw data was imported into GenABEL-native format (Aulchenko, Ripke, Isaacs, & van Duijn, 2007). All SNP-positions are given according to the dog CanFam3.1 assembly. Quality control (QC) was performed for each of the breeds separately, using R v3.2.1 and GenABEL v1.8-0. In the first step of QC we checked the individuals for potential duplicated samples. Then an iterative genotyping QC was performed, markers with minor allele frequency thresholds (MAF) below 5 % and call rates (SNP and individual call rates) below 95 % were removed, false discovery rates for Hardy-Weinberg equilibrium was set to 0.2. For each of the breeds, the correlation between phenotype and gender was checked, which was done by Fisher's exact test.

Genome-wide association analyses

A genome wide analysis was performed on the quality-controlled datasets for each breed included separately. An across-breed analysis was performed including a fixed effect for breed. For all the analyses, R v3.2.1 and GenABEL v1.8-0 were used. Using randomly selected autosomal markers, a genomic kinship matrix weighted by allele frequencies was computed in every breed. The genomic kinship matrix was used to perform multidimensional scaling (MDS) which projects genetic distance between individuals in two dimensions. In each breed, we used a standard linear mixed model, using the polygenic_hglm function from the hglm package (Rönnegård, Shen, & Alam, 2010). This method simultaneously accounts for population structure and kinship (Hoffman, 2013). For the Collie population, we used a K-means clustering to assign individuals to two subpopulations (rough and smooth collies). For each of breed-specific GWA study and the across-breed analysis, a quantile-quantile (QQ) and Manhattan plot was produced with R.

Meta-analysis of genome-wide association

A GWA meta-analysis of the five independent datasets was carried out using MetABEL v0.2.0 (Aulchenko et al., 2007). MetABEL performs a fixed effects meta-analysis assuming the associated shared allelic effect being the same in each dataset. Each study is weighted according to the inverse of its' squared standard error in order to maximize the power of discovery (Evangelou & Ioannidis, 2013). A Manhattan plot of the meta-analysis was created with the R package qqman (Turner, n.d.).

Allele frequencies

Allele frequencies was evaluated in the top ten SNPs from the breed specific analyses and in addition in a 500 kb area around a known candidate gene for anxiety (*DRD2*).

Results

Genome-wide association analyses

MDS plot of the breeds combined (n = 420) revealed 5 distinct breed populations (Figure 1a) and separate GWA analyses were performed for each breed respectively (Figure 1). MDS plot of the Collies (Figure 1b) showed two subpopulations, which represent rough and smooth collies. The quality control steps removed most of the SNPs due to MAF <0.05 or call rate < 95% and deviation from the Hardy-Weinberg equilibrium. The final data sets consisted of 81,453 markers in Collie, 96,472 in NSDTR, 109,402 in NB, 99,972 in ISWT and 106,163 in SB. In all the breeds except ISWT some dogs were removed due to low call rate (<95%) or because of too high identity by state (IBS) (>=0.95). No association was observed between phenotype and gender distribution in any of the breeds. The total number of dogs included in the final analyses can be found in Table 2.

[Figure 1]

[Table 2]

QQ-plots (Figure 2) in all the breeds showed an inflation factor λ around 1 indicating that the population stratification had been well controlled (Collie λ=1.03, ISWT λ=0.96, NSDTR λ=0.97, NB λ=0.96, SB λ=0.98). No markers reached a Bonferroni corrected 5 % significance level, (Table 3 and Figure 3). The top SNP in the Collie GWA analysis (BICF2P910427) was on chromosome 25 with P_{raw} 2.5x10^{-05}, in the ISWT analysis a marker on chromosome 15 (BICF2P1439743) had lowest nominal p-value (P_{raw} 1.3x10^{-04}). In the NSDTR analysis, the top SNP was found on chromosome 5 (BICF2P294742) (P_{raw} 1.9x10^{-04}). The top marker in the NB analysis was on chromosome 12 (TIGRP2P164812) with P_{raw} 1.1x10^{-04}, the SB analysis revealed the lowest p-value in the breed specific analyses with P_{raw} 5.3x10^{-05}, and the SNP was found on chromosome 7 (TIGRP2P91936). The position of the top ten SNPs in the breed specific analysis varied greatly between the breeds, markers on chromosome 7 was represented in all the breeds except NSDTR, but spanned across the chromosome; from position 16030057 in SB to 78596856 in ISWT, markers being closest in the Collie and and ISWT analyses (35352696 in ISWT and 48407230 in Collie). An across-breed analysis (92,141 markers, λ=0.95) was performed with breed as a fixed effect, the results in this analysis did not reveal genome-wide significance either, the top SNP (BICF2S23330151) was on chromosome 26 with P_{raw} 5.4x10^{-05} (Table 3).

[Figure 2]

[Figure 3]

Meta-analysis of genome-wide association

Meta-analysis was performed across breeds in order to identify a possible shared region. No genetic inflation was observed (λ=0.99) and the SNP with the lowest p-value was on chromosome 10 (BICF2P753483) (P_{raw} 2.37x10^{-05}) (Figure 4). The top SNP in this analysis was not among the top ten SNPs in either of the separate GWA analyses.

[Figure 4]

Allele frequencies

The allele frequencies of the top ten SNPs from each of the GWA analyses were compared to the other breeds included in the study and these varied considerably between the five breeds. The top SNP from the Collie GWA analysis BICF2P910427 on CFA 25 with major allele A and minor allele G had these frequencies of the major allele (cases versus controls frequencies in parenthesis); Collie 75% (87/62), across breeds 43% (46/40), ISWT 1% (0/2), NB 45% (46/44), NSDTR 9% (6/12) and SB 40% (39/42). An area of 500 kb around *DRD2* was also evaluated with regards to allele frequencies and there was a great difference in frequencies in most markers between the breeds; for some markers the frequency for the major allele was almost fixed in all the breeds. Other markers showed opposite frequency in cases and controls between breeds, e.g. in the marker BICF2S23153363 in the *DRD2* region the frequency of the major allele C in cases was 81% and 92% in controls in Collie, in ISWT the major allele frequency was higher in cases than in controls, 60% and 44% respectively. Frequencies for all the markers can be found in the supplementary tables.

[Table 3]

Discussion

In this genome-wide association study, no SNPs reached a genome wide significant association with anxiety. The SNP with the lowest p-value (P_{raw} 2.37x10^{-05}) was detected in the meta-analysis and was found on CFA10. The lowest p-value detected within a specific breed was P_{raw} 5.3 x10^{-05} (Staffordshire bull terrier analysis) on CFA7. This study shows the great variation in allele frequencies of many SNPs between different dog breeds.

Gene mapping of complex traits is considerably more difficult than mapping Mendelian traits. Findings from human studies have proposed that mental disorders like schizophrenia and bipolar disorders are most likely influenced by a large number of loci that are collectively responsible for variation in risk (International Schizophrenia Consortium et al., 2009). Even though the heritabilities for these traits tend to be high, the identification of single genes has been difficult and huge sample sizes have been needed to be able to obtain statistical significance in human studies. Mapping disease genes using GWAS in dogs requires approximately 10x fewer markers and samples than in human populations as the breeds represent genetic isolates with long LD and low genetic heterogeneity. However, population structure, cryptic relatedness, and extensive regions of near fixation in breeds have complicated GWAS analysis and few studies have successfully mapped risk factors for complex, multigenic diseases (Karlsson et al., 2013; Wilbe et al., 2010).

The genome-wide association analyses in this study showed significant differences in allele frequencies of many SNPs between breeds. The top 10 SNPs within the breed specific analyses showed that some SNPs could have opposite allele frequencies in cases/controls in the different breeds included. Even if there was a nominal significance in each breed, a combined analysis could therefore mask some of the effect. The lowest nominal significance obtained for a single SNP was 5.3 $x10^{-05}$ (Staffordshire bull terrier analysis), however none of the SNPs showed genome-wide significant markers. Due to the close linkage of many SNP markers and the high number of markers analyzed in SNP arrays, Bonferroni correction may be too conservative (Sutter, 2004). Studies in humans have shown that Bonferroni testing can lead to a decreased ability for association studies to detect truly associated markers (Belonogova, Svishcheva, van Duijn, Aulchenko, & Axenovich, 2013a). In addition, the ability to detect modest genetic effects can be difficult. Because of these drawbacks with GWAS studies, gene- or region-based analysis of genome-wide association studies have been proposed (Belonogova, Svishcheva, van Duijn, Aulchenko, & Axenovich, 2013b) (Beyene, Tritchler, Asimit, & Hamid, 2009).

This study included over 400 dogs, and there are several examples that complex disorders in dogs can be mapped with around 100 cases and 100 controls (Bianchi et al., 2015; Forsberg et al., 2015; Wilbe et al., 2010) (Bannasch et al., 2010). Mendelian traits have been mapped with genome-wide association analysis in dogs with as low as ~20 dogs (Karlsson et al., 2007). Since the 187 cases and 216 controls in this study population consists of samples from five unrelated breeds, the study is likely to be underpowered, even with a relative large number of dogs included. The deflated QQ-plots could also point to too few samples. Too few samples would give low statistical power and for further investigation it is desired to increase the number of dogs genotyped. The difficulty of achieving genome-wide significance in a study including several breeds can also be illustrated by the difference in allele frequencies between the breeds (supplementary tables). Human studies have found that variations in gene allele frequencies can contribute to differences in the prevalence of common complex diseases among populations (Mattei et al., 2008) (Goddard, Hopkins, Hall, & Witte, 2000).

In our study the top SNP from the meta-analysis showed the lowest p-value of all the analyses (P_{raw} $2.37\text{x}10^{-05}$) and was on chromosome 10. This marker was however not among the top 10 SNPs in any of the breed-specific analyses, and thus no common shared region between the breeds could be identified. This also further suggests that the genetic risk alleles for the phenotype studied could be different between the dog breeds included, or that different SNP-alleles could be syntenic to the functional gene in different breeds.

Heritable changes in gene expression and cellular function not involving alterations in the DNA could also play a role in behavioral disorders. Epigenetic mechanisms include many types of histone and DNA modifications as well as alterations in many types of non-histone proteins and non-coding RNAs. There are increasing evidence that such mechanisms are important effectors in psychiatric conditions (Nestler, Peña, Kundakovic, Mitchell, & Akbarian, 2015). In this study a few (4) microRNAs was present in the area around the top 10 SNPs in the breed specific analyses.

In summary, we did not find genome-wide association of SNPs to noise sensitivity in the separate breeds, neither in the across breed analysis. We believe that the most likely reason is that the study is underpowered. An across breed analysis may also be suboptimal to detect associations in a GWAS study because of linked SNPs, and that the breeds could have different marker-functional-gene haplotypes. In situations with limited size of samples, candidate gene studies using SNPs within specific candidate genes would probably be a better alternative to detect associations to behavioral traits. The study also confirms that a GWAS of a complex trait across unrelated breeds probably does not add power due to genetic heterogeneity and different haplotype phases.

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Tables and Figures

Table 1 Breeds and number of dogs included in the study

Breed (abbreviation)	Number of dogs (females, males)
Collie	98 (66, 32)
Irish softcoated wheaten terrier (ISWT)	44 (27, 17)
Nova Scotia duck tolling retriever (NSDTR)	33 (17, 16)
Norwegian Buhund (NB)	108 (56, 52)
Staffordshire bull terrier (SB)	137 (72, 65)
Total	**420 (238, 182)**

Table 2 Number of dogs included after quality control steps

Breed (abbreviation)	Number of dogs (cases, controls)
Collie	93 (49, 44)
Irish softcoated wheaten terrier (ISWT)	44 (20, 24)
Nova Scotia duck tolling retriever (NSDTR)	32 (16, 16)
Norwegian Buhund (NB)	104 (36, 68)
Staffordshire bull terrier (SB)	130 (66, 64)
Total	**403 (187, 216)**

Table 3 Top 10 SNPs from each breed specific GWAS and across-breed analysis

SNP	Chr	Position	P_{raw}	A1	A2	Region	Size(kb)	Genes
Collie								
BICF2P910427	25	47043919	2.5e-04	A	G	46943919-47053919	110	GBX2, ASB18, AGAP1
BICF2P1298754	7	48407230	3.8e-04	A	G	48307230-48407230	100	None
BICF2S23044599	7	48442826	3.8e-04	T	C	48342826-48442826	100	None
BICF2P1453413	7	48455845	3.8e-04	C	T	48355845-48455845	100	None
BICF2P1208583	7	49099379	3.8e-04	A	G	48099379-49099379	1,000	PIK3C3, NUDT21
BICF2P9780	7	49115454	3.8e-04	C	T	49115257-50115257	1,000	None
BICF2P9787	7	49117856	3.8e-04	T	C	49115257-50115257	1,000	None
BICF2G630302393	11	58213247	6.2e-04	G	A	58200000-59114276	1,000	RNF20, GRIN3A, TMEM246
BICF2P197380	2	75050435	6.5e-04	G	A	75050435-76050435	1,000	Many; NCMAP, RCAN3, NIPAL3, STPG1, GRHL3
TIGRP2P30887	2	75057538	6.5e-04	G	A	75050435-76050435	1,000	As above
ISWT								
BICF2P1439743	15	3276885	1.3e-04	G	A	3276885-3476885	1,000	SNORA55, MACF1
BICF2P1279311	25	37111073	2.0e-04	T	C	37111073-39111073	2,000	None
TIGRP2P199474	15	33089790	2.1e-04	C	T	33089790-35089790	2,000	None
TIGRP2P199550	15	33306096	2.1e-04	G	T	33306096-35306096	2,000	None
BICF2P377493	7	78596856	3.3e-04	C	A	78596856-81596856	2,000	None
BICF2P1170958	37	17690997	5.1e-04	A	G	17690997-19690997	2,000	None
BICF2S23051548	7	35352696	5.6e-04	G	A	35352696-37352696	2,000	MIR7180
TIGRP2P95152	7	35517809	5.6e-04	G	C	35352696-37352696	2,000	MIR7180
TIGRP2P95153	7	35520789	5.6e-04	G	A	35520789-37520789	2,000	MIR7180
BICF2S23613834	7	35561591	5.6e-04	C	T	35561591-37561591	2,000	MIR7180
NSDTR								
BICF2P294742	5	72929885	1.9e-04	T	C	72929885-74929885	2,000	None
BICF2P833251	8	27067240	1.9e-04	A	G	27067240-31067240	4,400	PYGL, PTGER2, BMP4, LGALS3
BICF2P938344	8	27087764	1.9e-04	T	C	27087764-31087764	4,400	As above
BICF2S23256667	8	28705740	1.9e-04	C	T	28705740-32705740	4,400	As above
BICF2P1250869	8	29805372	1.9e-04	A	T	29805372-33805372	4,400	As above
BICF2P241039	8	29816886	1.9e-04	A	G	29816886-33816886	4,400	As above

BICF2P1277877	8	30502653	1.9e-04	A	C	30502653-34502653	4,400	As above
BICF2P616056	8	30519307	1.9e-04	C	A	30519307-34519307	4,400	As above
BICF2P971817	8	30547677	1.9e-04	G	A	30547677-34547677	4,400	As above
BICF2S22940011	8	30552185	1.9e-04	G	T	30552185-34552185	4,400	As above
NB								
TIGRP2P164812	12	41201459	1.1e-04	A	G	41201459-43201459	2,000	None
BICF2P147370	12	52026506	2.2e-04	C	T	52026506-54026506	2,000	None
BICF2G630109464	16	26795472	4.6e-04	G	A	26795472-28795472	2,000	STAR, ADRB3
BICF2G630109763	16	27453414	4.6e-04	T	C	27453414-29453414	2,000	STAR, ADRB3
BICF2S23442141	14	10462378	4.8e-04	G	A	10462378-12462378	2,000	None
BICF2G630521054	14	10503040	4.8e-04	A	G	10503040-12503040	2,000	None
BICF2S233677	12	49559697	5.1e-04	T	A	49559697-51559697	2,000	None
BICF2P518261	16	27036665	5.3e-04	T	A	27036665-29036665	2,000	STAR, ADRB3
BICF2S23214294	7	51161290	5.6e-04	T	C	51161290-53161290	2,000	None
BICF2P1031887	12	48313005	6.5e-04	A	G	48313005-51313005	2,000	None
SB								
TIGRP2P91936	7	16030057	5.3e-05	G	T	16030057-18030057	2,000	None
BICF2S23237174	35	23763305	1.1e-04	A	T	23763305-25763305	2,000	None
BICF2P1046706	2	80308746	2.2e-04	G	C	80308746-82308746	2,000	PADI6, SDHD, CASP9
BICF2P1303211	2	80489302	2.3e-04	G	T	80489302-82489302	2,000	PADI6, SDHD, CASP9
BICF2P539807	34	40834516	2.3e-04	A	G	40834516-42834516	2,000	NONE
BICF2P480550	11	68646419	2.3e-04	G	A	68646419-71646419	2,000	TNC
BICF2S24317378	11	57423231	2.3e-04	G	A	57423231-59423231	2,000	None
BICF2G630301833	11	57427458	2.7e-04	G	A	57427458-59427458	2,000	None
BICF2P530778	4	58792307	2.8e-04	A	C	58792307-61792307	2,000	PDGFRB, PDE6A, ADRB2, MIR143/145/378
BICF2G630219062	17	12427593	2.9e-04	G	A	12427593-13427593	1,000	RAD51AP2,SMC6, GEN1, MSGN1, KCNS3
SNP	Chr	Position	P_{raw}	A1	A2	Region	Size(kb)	Genes
Across all breeds								
BICF2S23330151	26	17463697	5.4e-05	G	A	17463697-19463697	2,000	Many, MVK, MMAB, UBE3B, KCTD10, TMEM119
BICF2G630414271	8	57859452	5.6e-05	G	A	57859452-59859452	2,000	GALC
BICF2P822147	10	7658201	6.0e-05	C	A	7658201-7858201	2,000	None
BICF2P544714	11	61707006	6.5e-05	G	A	61707006-63707006	2,000	None
BICF2P460899	31	15757769	1.4e-04	G	A	15757769-17757769	2,000	None
BICF2S23012887	10	8059173	1.5e-04	C	A	8059173-8259173	2,000	None
BICF2P1438249	11	61718412	1.8e-04	G	T	61718412-63718412	2,000	None
BICF2P1270191	26	16303070	2.2e-04	T	C	16303070-18303070	2,000	OASL, TRPV4
BICF2P517106	28	32050843	2.5e-04	A	G	32050843-34050843	2,000	None
BICF2P271175	28	32067057	2.5e-04	T	C	32067057-34067057	2,000	None

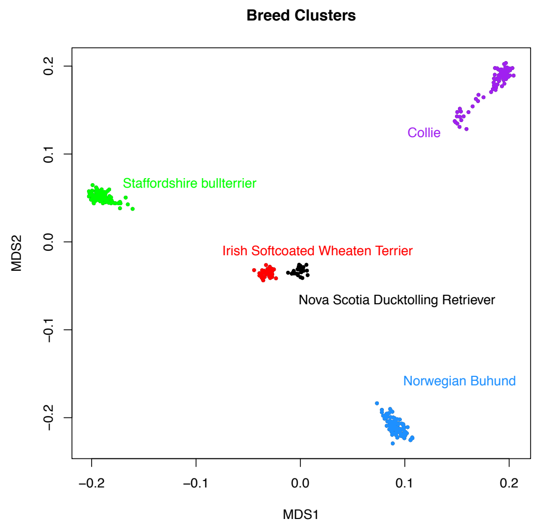
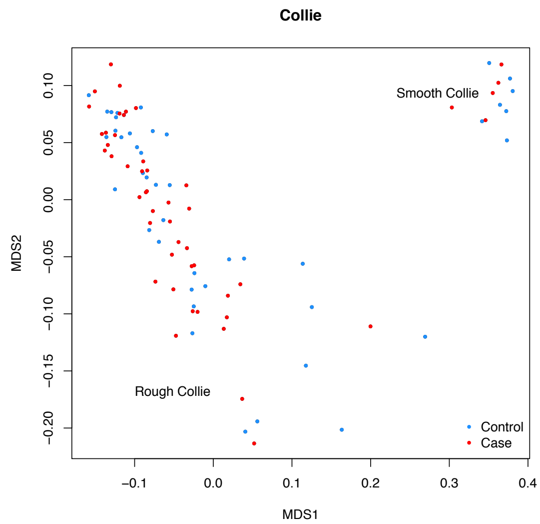


Figure 1a



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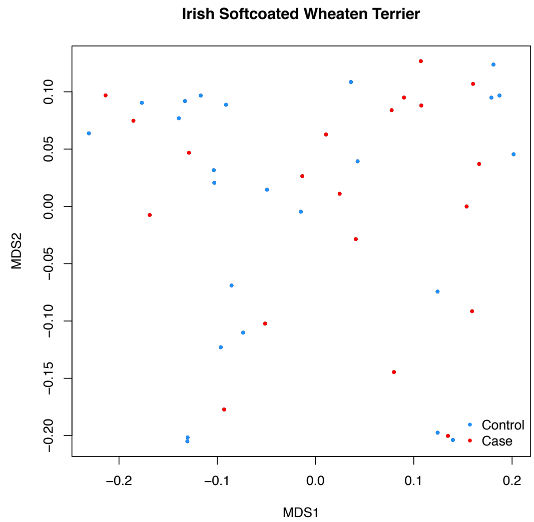

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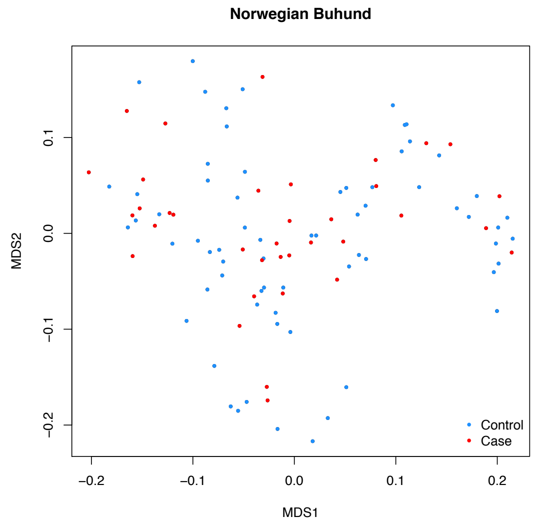

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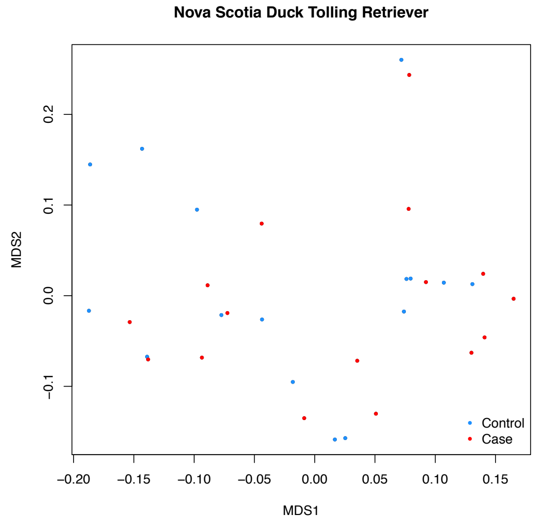

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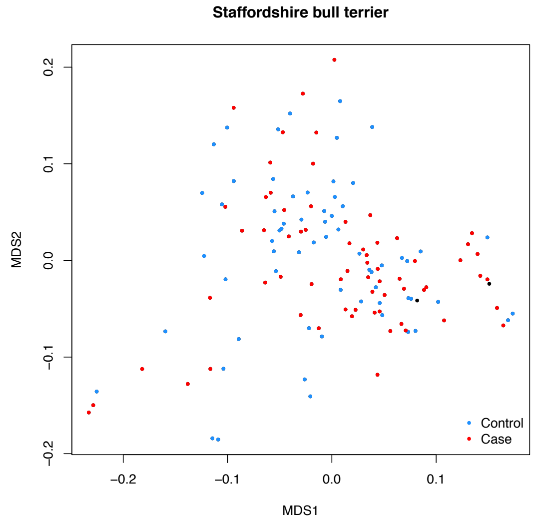


f

Figure 1: MDS plot of the five breeds showing distinct genetic populations (1a) and the breed specific MDS plots showing homogenous sample sets (1c-f), except for the Collie (1b) which illustrate the division into two populations.

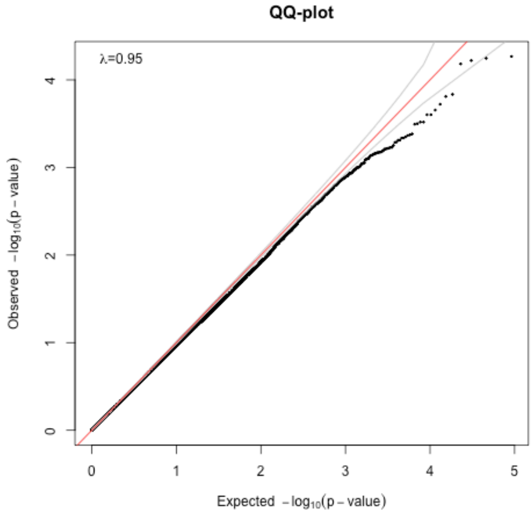
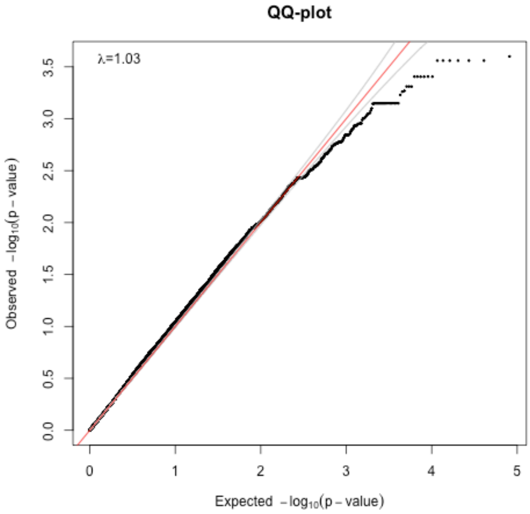
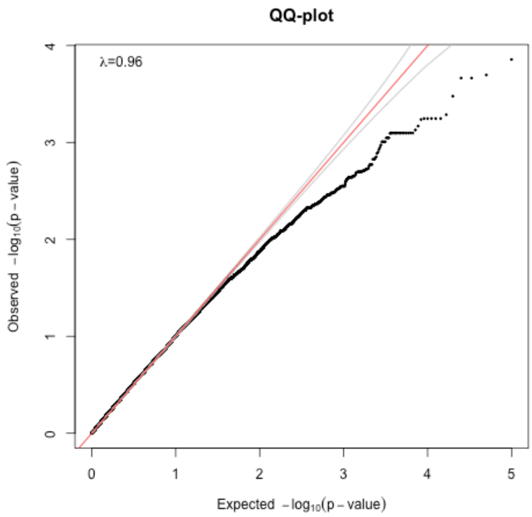


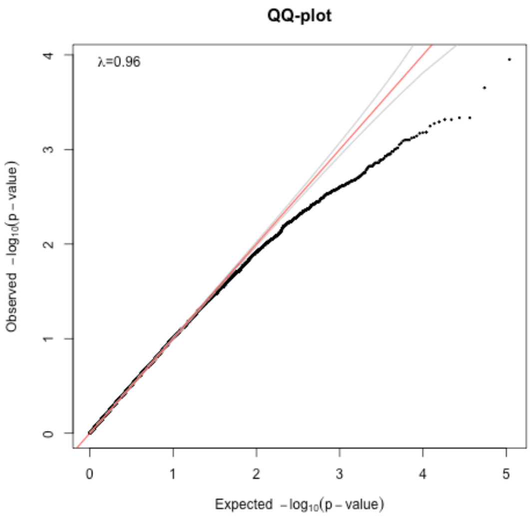
Figure 2a Across breed



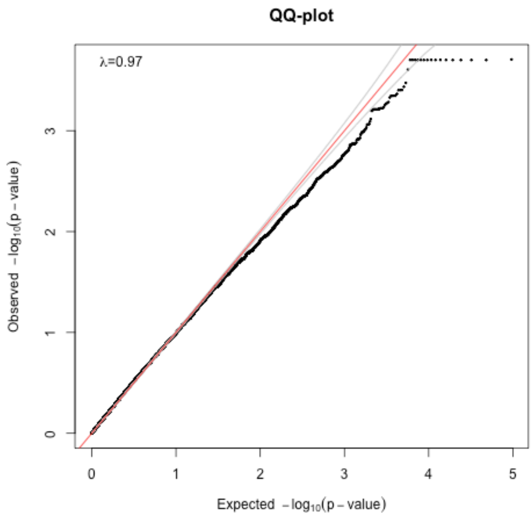
b Collie



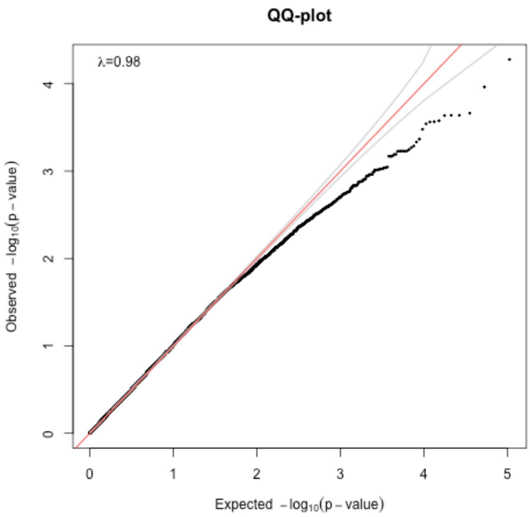
c ISWT



d Norwegian Buhund



e NSDTR



f Staffordshire bull terrier

Figure 2: QQ plots showing no inflation after the mixed model approach in across breed analysis λ=0.95 (a), Collie λ=1.03 (b), ISWT λ=0.96 (c), NB λ=0.96 (d), NSDTR λ=0.97 (e) and SB λ=0.98 (f). Theoretical p-values are plotted against observed p-values (mixed model), grey lines denote 5% and 95% confidence intervals.

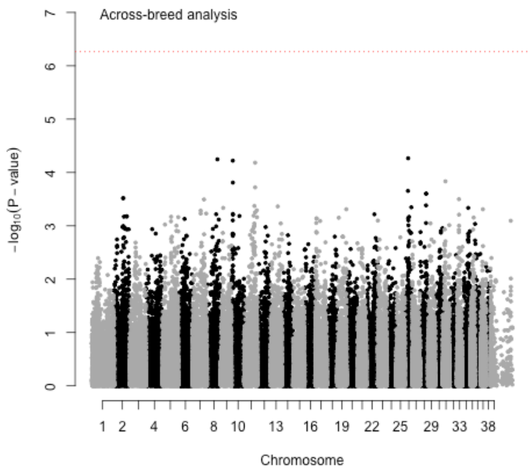
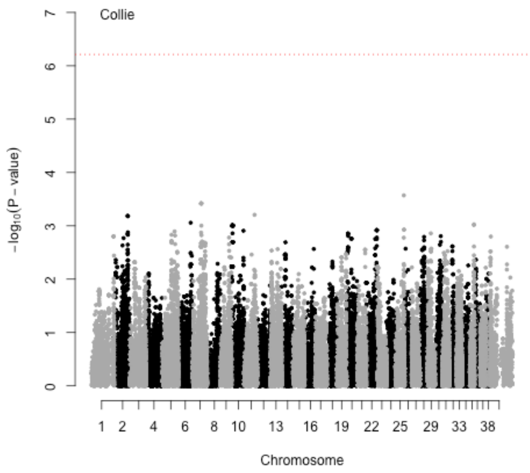


Figure 3a



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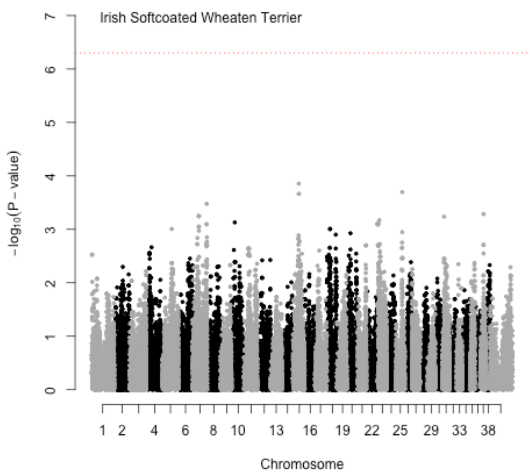
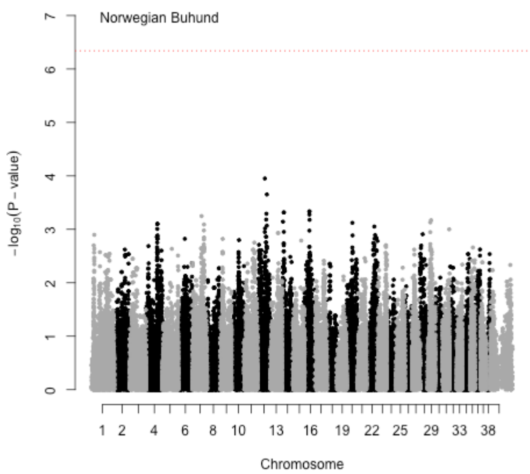


c



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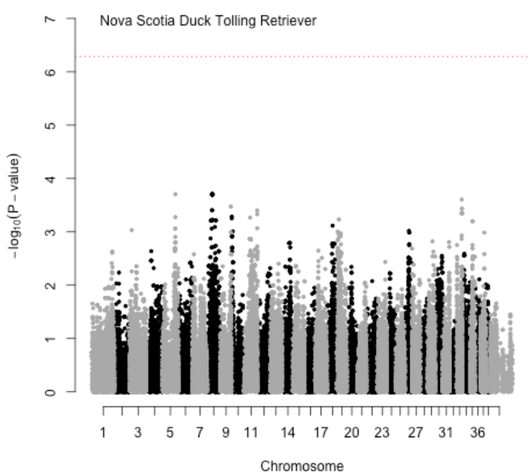
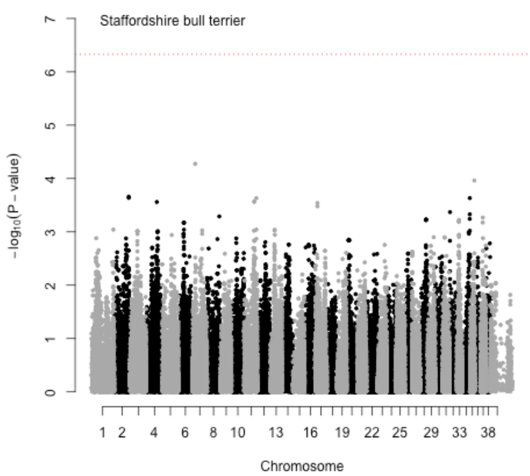


e



f

Figure 3: Manhattan plots with Bonferroni corrected p-value of 0.05 marked as dashed red line.

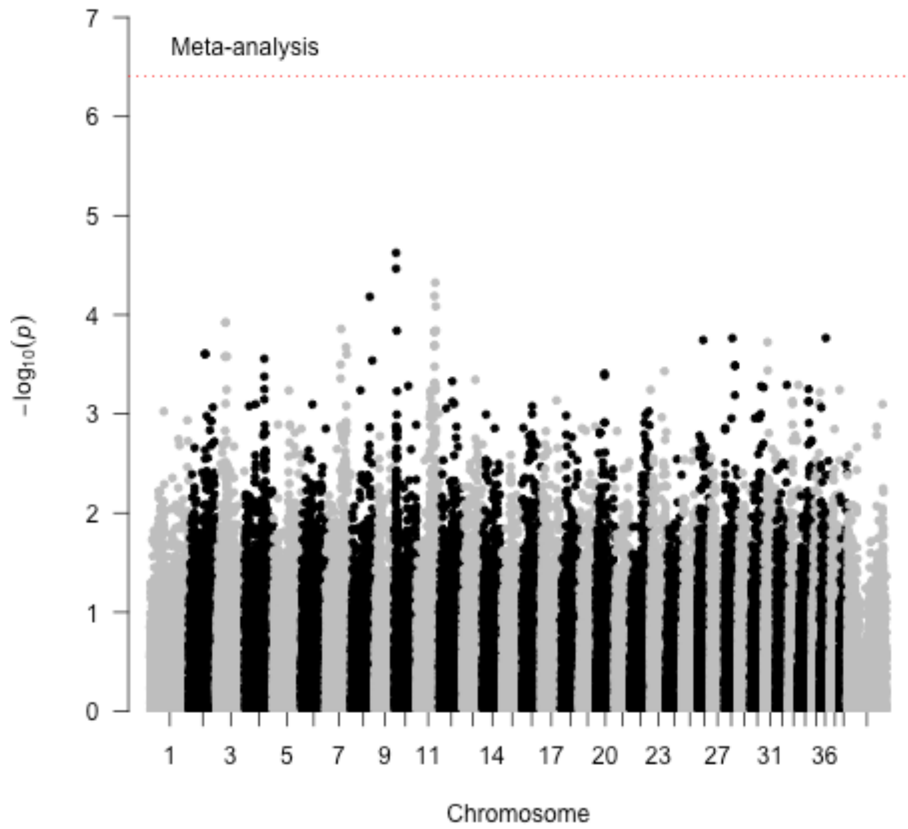


Figure 4: Manhattan plot from the multi-breed meta-analysis with Bonferroni corrected p-value of 0.05 marked as dashed red line.

Supplementary tables

Allele frequencies (%) of major allele (in bold) with allele frequencies in cases and controls of the top 10 SNPs from the across breed analysis compared to the other breeds.

Marker (Alleles) Position	Chr	**Across breeds** (F_A/F_U)	Collie (F_A/F_U)	ISWT (F_A/F_U)	NB (F_A/F_U)	NSDTR (F_A/F_U)	SB (F_A/F_U)
BICF2S23330151 (**G**/A) 17463697	26	83 (89/77)	98 (100/98)	74 (80/69)	74 (82/70)	84 (88/81)	80 (87/72)
BICF2G630414271 (**G**/A) 57859452	8	80 (86/75)	57 (66/47)	92 (95/90)	83 (88/81)	95 (97/94)	88 (95/80)
BICF2P822147 (**C**/A) 7658201	10	78 (84/73)	89 (96/81)	64 (78/52)	87 (92/84)	92 (97/88)	65 (70/61)
BICF2P544714 (**G**/A) 61707006	11	68 (75/61)	60 (65/53)	94 (95/94)	50 (61/44)	55 (69/40)	81 (87/76)
BICF2P460899 (**G**/A) 15757769	31	68 (60/75)	47 (36/59)	41 (25/54)	96 (96/96)	47 (44/50)	75 (72/78)
BICF2S23012887 (**C**/A) 8059173	10	82 (88/77)	100 (100/100)	100 (100/100)	78 (86/74)	59 (75/44)	72 (78/65)
BICF2P1438249 (**C**/A) 61718412	11	70 (77/63)	60 (65/53)	100 (100/100)	100 (100/100)	59 (72/47)	83 (88/78)
BICF2P1270191 (**T**/C) 16303070	26	87 (90/83)	96 (98/93)	100 (100/100)	96 (97/95)	59 (72/47)	75 (83/68)
BICF2P517106 (**A**/G) 32050843	28	94 (98/91)	100 (100/100)	95 (98/94)	93 (97/90)	77 (81/72)	95 (100/90)
BICF2P271175 (**T**/C) 32067057	28	94 (98/91)	100 (100/100)	95 (98/94)	93 (97/90)	77 (81/72)	95 (100/90)

Chr Chromosome
F_A Allele frequency in cases
F_U Allele frequency in controls
ISWT Irish softcoated wheaten terrier
NB Norwegian buhund
NSDTR Nova scotia duck tolling retriever
SB Staffordshire bull terrier

Allele frequencies (%) of major allele (in bold) with allele frequencies in cases and controls of the top 10 SNPs from the Collie GWA analysis compared to the other breeds.

Marker (Alleles) Position	Chr	**Collie** (F_A/F_U)	Across breeds (F_A/F_U)	ISWT (F_A/F_U)	NB (F_A/F_U)	NSDTR (F_A/F_U)	SB (F_A/F_U)
BICF2P910427 (**A**/G) 47043919	25	75 (87/62)	43 (46/40)	1 (0/2)	45 (46/44)	9 (6/12)	40 (39/42)
BICF2P1298754 (**A**/G) 48407230	7	99 (100/97)	89 (89/89)	100 (100/100)	99 (100/98)	100 (100/100)	98 (95/100)
BICF2S23044599 (**T**/C) 48442826	7	88 (94/80)	68 (74/62)	81 (88/75)	23 (21/25)	86 (94/79)	80 (80/78)
BICF2P1453413 (**C**/T) 48455845	7	88 (94/80)	67 (74/61)	81 (88/75)	19 (16/21)	91 (100/82)	80 (81/79)
BICF2P1208583 (**A**/G) 49099379	7	88 (94/80)	69 (75/63)	88 (93/83)	17 (21/15)	52 (53/50)	94 (91/98)
BICF2P9780 (**C**/T) 49115454	7	88 (94/80)	69 (75/63)	88 (93/83)	18 (22/15)	52 (53/50)	94 (91/98)
BICF2P9787 (**T**/C) 49117856	7	88 (94/80)	67 (73/62)	69 (68/71)	18 (22/15)	52 (53/50)	94 (91/98)
BICF2G630302393 (**G**/A) 58213247	11	82 (72/92)	77 (71/82)	93 (95/92)	100 (100/100)	29 (41/18)	61 (55/67)
BICF2P197380 (**C**/T) 75050435	2	85 (94/74)	42 (48/37)	18 (23/15)	0 (0/0)	59 (66/53)	50 (45/55)
TIGRP2P30887 (**G**/A) 75057538	2	85 (94/74)	32 (26/37)	78 (80/77)	40 (38/41)	41 (50/32)	56 (53/59)

Chr Chromosome
F_A Allele frequency in cases
F_U Allele frequency in controls
ISWT Irish softcoated wheaten terrier
NB Norwegian buhund
NSDTR Nova scotia duck tolling retriever
SB Staffordshire bull terrier

Allele frequencies (%) of major allele (in bold) with allele frequencies in cases and controls of the top 10 SNPs from the ISWT GWA analysis compared to the other breeds.

Marker (Alleles) Position	Chr	**ISWT** (F_A/F_U)	Across breeds (F_A/F_U)	Collie (F_A/F_U)	NB (F_A/F_U)	NSDTR (F_A/F_U)	SB (F_A/F_U)
BICF2P1439743 (**G**/A) 32768856	15	57 (83/35)	73 (77/70)	94 (93/95)	59 (59/59)	6 (6/6)	92 (91/93)
BICF2P1279311 (**T**/C) 37111073	25	53 (30/73)	42 (41/43)	4 (4/4)	16 (15/17)	8 (9/6)	93 (92/95)
TIGRP2P199474 (**C**/T) 33089790	15	65 (88/46)	94 (96/91)	93 (92/94)	100 (100/100)	91 (97/85)	100 (100/100)
TIGRP2P199550 (**G**/T) 33306096	15	65 (88/46)	66 (64/67)	9 (9/10)	100 (100/100)	85 (91/79)	74 (72/76)
BICF2P377493 (**C**/A) 78596856	7	67 (90/48)	29 (29/29)	6 (5/8)	26 (24/28)	48 (44/53)	29 (28/30)
BICF2P1170958 (**A**/G) 17690997	37	59 (40/75)	79 (79/78)	68 (73/62)	73 (77/81)	72 (69/76)	99 (99/99)
BICF2S23051548 (**G**/A) 35352696	7	76 (93/63)	83 (85/81)	96 (97/94)	79 (76/81)	59 (56/62)	85 (87/83)
TIGRP2P95152 (**G**/C) 35517809	7	76 (93/63)	71 (75/69)	100 (100/100)	57 (56/57)	89 (94/85)	54 (57/53)
TIGRP2P95153 (**G**/A) 35520789	7	76 (93/63)	68 (71/65)	90 (93/87)	57 (58/57)	86 (88/85)	51 (53/50)
BICF2S23613834 (**C**/T) 35561591	7	76 (93/63)	74 (76/72)	100 (100/100)	62 (60/62)	89 (94/85)	57 (58/57)

Chr	Chromosome
F_A	Allele frequency in cases
F_U	Allele frequency in controls
ISWT	Irish softcoated wheaten terrier
NB	Norwegian buhund
NSDTR	Nova scotia duck tolling retriever
SB	Staffordshire bull terrier

Allele frequencies (%) of major allele (in bold) with allele frequencies in cases and controls of the top 10 SNPs from the NB GWA analysis compared to the other breeds.

Marker (Alleles) Position	Chr	**NB** (F_A/F_U)	Across breeds (F_A/F_U)	Collie (F_A/F_U)	ISWT (F_A/F_U)	NSDTR (F_A/F_U)	SB (F_A/F_U)
TIGRP2P164812 (**A**/G) 41201459	12	76 (65/82)	71 (70/73)	98 (100/97)	17 (10/23)	48 (56/41)	72 (70/73)
BICF2P147370 (**C**/T) 52026506	12	82 (73/87)	59 (53/65)	20 (16/25)	90 (90/90)	62 (56/68)	58 (57/59)
BICF2G630109464 (**G**/A) 26795472	16	70 (60/75)	89 (89/90)	99 (99/100)	94 (95/94)	74 (75/74)	100 (100/100)
BICF2G630109763 (**T**/C) 27453414	16	70 (60/75)	57 (55/59)	96 (94/98)	72 (78/67)	47 (44/50)	18 (20/16)
BICF2S23442141 (**G**/A) 10462378	14	71 (63/76)	79 (77/81)	79 (94/98)	35 (38/33)	97 (94/100)	94 (91/97)
BICF2G630521054 (**A**/G) 10503040	14	71 (63/76)	77 (74/80)	100 (100/100)	63 (63/63)	97 (94/100)	66 (61/71)
BICF2S233677 (**T**/A) 49559697	12	83 (77/87)	88 (88/89)	100 (100/100)	68 (63/73)	47 (50/44)	100 (100/100)
BICF2P518261 (**T**/A) 27036665	16	64 (53/70)	71 (70/73)	61 (64/57)	88 (83/92)	55 (59/50)	84 (83/86)
BICF2S23214294 (**T**/C) 51161290	7	36 (50/27)	52 (55/50)	55 (51/59)	38 (38/38)	35 (41/29)	74 (69/79)
BICF2P1031887 (**A**/G) 48313005	12	91 (83/95)	95 (94/95)	100 (100/100)	95 (100/92)	94 (91/97)	94 (96/93)

Chr Chromosome
F_A Allele frequency in cases
F_U Allele frequency in controls
ISWT Irish softcoated wheaten terrier
NB Norwegian buhund
NSDTR Nova scotia duck tolling retriever
SB Staffordshire bull terrier

Allele frequencies (%) of major allele (in bold) with allele frequencies in cases and controls of the top 10 SNPs from the SB GWA analysis compared to the other breeds.

Marker (Alleles) Position	Chr	**SB** (F_A/F_U)	Across breeds (F_A/F_U)	Collie (F_A/F_U)	ISWT (F_A/F_U)	NB (F_A/F_U)	NSDTR (F_A/F_U)
TIGRP2P91936 (**G**/T) 16030057	7	84 (73/94)	50 (47/52)	25 (22/28)	34 (38/31)	42 (44/41)	33 (38/28)
BICF2S23237174 (**A**/T) 23763305	35	78 (91/75)	87 (96/81)	98 (100/97)	100 (100/100)	68 (84/63)	98 (100/97)
BICF2P1046706 (**G**/C) 80308746	2	76 (86/66)	57 (60/54)	52 (50/53)	7 (8/6)	58 (55/60)	58 (63/53)
BICF2P1303211 (**G**/T) 80489302	2	82 (91/74)	69 (73/66)	80 (83/77)	77 (68/85)	57 (55/59)	12 (9/15)
BICF2P539807 (**A**/G) 40834516	34	53 (40/67)	61 (57/63)	99 (99/100)	67 (58/75)	39 (38/39)	38 (44/32)
BICF2P480550 (**G**/A) 68646419	11	86 (93/78)	71 (75/67)	92 (94/90)	84 (80/88)	31 (27/34)	56 (47/65)
BICF2S24317378 (**G**/A) 57423231	11	78 (88/68)	60 (64/56)	26 (33/17)	72 (78/69)	59 (59/59)	70 (56/82)
BICF2G630301833 (**G**/A) 57427458	11	78 (88/68)	61 (67/57)	26 (33/18)	72 (78/69)	59 (59/59)	89 (88/91)
BICF2P530778 (**A**/C) 58792307	4	64 (76/51)	70 (73/68)	38 (38/38)	100 (100/100)	86 (88/85)	100 (100/100)
BICF2G630219062 (**G**/A) 12427593	17	91 (84/98)	87 (85/90)	75 (78/72)	74 (70/77)	98 (99/98)	91 (91/91)

Chr Chromosome
F_A Allele frequency in cases
F_U Allele frequency in controls
ISWT Irish softcoated wheaten terrier
NB Norwegian buhund
NSDTR Nova scotia duck tolling retriever
SB Staffordshire bull terrier

Allele frequencies (%) of major allele (in bold) with allele frequencies in cases and controls from markers in a 500 kb region around *DRD2* on canine chromosome 5.

Marker (Alleles) Position	Across breeds (F_A/F_U)	Collie (F_A/F_U)	ISWT (F_A/F_U)	NB (F_A/F_U)	NSDTR (F_A/F_U)	SB (F_A/F_U)
BICF2P1443513 (**G**/A) 19538710	98 (97/98)	100 (100/100)	100 (100/100)	100 (100/100)	72 (69/75)	100 (100/100)
BICF2P231732 (**C**/T) 19584747	98 (97/98)	100 (100/100)	100 (100/100)	100 (100/100)	72 (69/75)	100 (100/100)
G331F25S214 (**A**/G) 19622983	91 (89/92)	100 (100/100)	91 (88/94)	100 (100/100)	73 (78/69)	81 (78/84)
BICF2S23048457 (**G**/C) 19666262	97 (97/97)	100 (100/100)	98 (98/98)	98 (100/97)	72 (69/75)	100 (100/100)
BICF2S23153363 (**C**/T) 19677807	73 (71/75)	86 (81/92)	51 (60/44)	95 (95/96)	66 (69/63)	55 (54/56)
BICF2P532373 (**A**/G) 19695809	87 (87/87)	58 (60/57)	98 (98/98)	98 (100/97)	100 (100/100)	93 (94/92)
BICF2P1359630 (**C**/A) 19705966	99 (99/99)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)	96 (97/95)
BICF2S23657115 (**T**/C) 19730821	94 (95/94)	100 (100/100)	91 (88/94)	100 (100/100)	72 (78/66)	92 (94/89)
BICF2P889900 (**C**/T) 19740100	70 (69/71)	63 (33/41)	77 (80/75)	68 (68/68)	64 (56/72)	95 (96/94)
BICF2P548123 (**C**/A) 19750707	98 (99/98)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)	96 (97/95)
BICF2P905138 (**C**/G) 19766934	62 (61/63)	63 (33/41)	68 (68/69)	70 (69/70)	42 (44/41)	76 (79/72)
BICF2S22964504 (**C**/T) 19774068	53 (53/54)	64 (33/41)	80 (10/27)	59 (97/98)	42 (44/41)	75 (78/71)
BICF2S23126854 (**T**/C) 19790332	61 (61/61)	86 (81/92)	20 (10/27)	59 (59/58)	42 (44/41)	63 (67/59)
BICF2P251646 (**C**/T) 19830043	86 (87/85)	100 (100/100)	80 (83/77)	94 (96/93)	28 (31/25)	85 (87/84)

BICF2P783886 (**T**/C) 19860243	55 (47/59)	62 (34/42)	42 (30/52)	91 (92/91)	40 (41/41)	45 (57/54)
BICF2P573421 (**G**/A) 19866781	97 (97/97)	100 (100/100)	100 (100/100)	100 (100/100)	69 (72/66)	99 (99/100)
BICF2P788683 (**A**/G) 19903695	63 (63/63)	100 (100/100)	26 (23/29)	62 (59/63)	70 (69/72)	49 (49/48)
BICF2P1330944 (**G**/T) 19911740	81 (82/80)	100 (100/100)	100 (100/100)	55 (49/59)	70 (69/72)	85 (86/84)
BICF2P907815 (**T**/C) 19923429	58 (58/58)	100 (100/100)	47 (40/52)	53 (46/57)	40 (43/38)	40 (42/38)
BICF2P356602 (**G**/A) 19964113	96 (96/97)	100 (100/100)	100 (100/100)	100 (100/100)	100 (100/100)	89 (90/88)
BICF2P667205 (**C**/T) 19980521	91 (91/91)	100 (100/100)	86 (93/81)	100 (100/100)	100 (100/100)	78 (78/78)
BICF2P797660 (**T**/C) 19994864	52 (49/55)	40 (36/44)	35 (33/38)	66 (62/68)	100 (100/100)	45 (45/45)

DRD2 IS ASSOCIATED WITH ANXIETY IN SOME DOG BREEDS

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Abstract

Behavioural problems occur frequently in dogs and represent a significant threat to dog welfare. Anxiety, phobias and fears make up a large portion of these issues. Behavioural traits tend to have high heritability estimates and show strong selection response in breeding. Neurotransmitters, like dopamine, adrenaline, noradrenaline, serotonin and others, are known to influence emotion and behaviour. Correct regulation of these transmitters is crucial for optimal neurological and mental function and various transporters and receptors work together in complex interaction to maintain this regulation. The DRD2 gene encodes the dopamine receptor 2. Association between the DRD2 gene and generalized anxiety in Havanese dogs, expressed both through observation by an external evaluator and through owner questionnaires, was investigated. We found significant association between two SNPs in exon 2 of the DRD2 gene and generalized anxiety. Because anxiety related behaviour have been shown to commonly co-occur, the two SNPs were also investigated for possible association to noise sensitivity in 5 breeds. Significant association was detected between DRD2 and noise sensitivity in the Irish softcoated wheaten terrier and Collie.

Introduction

Behavioural problems occur frequently in dogs (2) (5) (20) and represent a significant threat to dog welfare. Behavioural problems are an important cause of both dog abandonment (22) and euthanasia (9). In a study from the United States, at least one behavioural reason was recorded for 40% of relinquished dogs and behavioural reasons accounted for 27% of single-reason canine relinquishments (22). It is estimated that behavioural problems account for 10-15% of all euthanasias of dogs and cats in North America (14).

Various types of anxiety constitute a large portion of these behavioural issues and include generalized anxiety disorders, phobias, separation anxiety, noise sensitivity and fear. One study (2) showed that anxiety, phobias and fears make up well over 20% of cases presented at a large behavioural clinic. Fear aggression towards owners (5.2%) and strangers (16.8%) were common complaints as well. In addition to the fact that anxiety represent a welfare issue in itself, anxious dogs might also be subject to secondary welfare issues such as isolation or unethical training methods. Anxiety issues are also of relevance to society, as aggression resulting from anxiety can create unpleasant or dangerous situations for people or other dogs. Several studies have found that different types of anxiety commonly co-occur (19) (26).

There is a general acceptance that behavioural traits tend to have high heritabilities in both laboratory animals, humans and many other species. High genetic influence has been detected for personality traits of shyness, inhibition and fear in people (5) and heritability estimates for generalized anxiety disorders in humans are often high (3) (8) (25). One study (21), on behavioural traits in dogs estimated the heritability of the shyness/boldness aspect of a dog's personality to be 0.25. Heritability estimates of fearfulness was found to be 0.5 in one study of guide dogs (6) and in a study of Rough collies, fearlessness was estimated to be 0.20 (1). A study including Labrador retrievers found that gun shyness had a heritability estimate of 0.56 (28).

In a well-known selection study in foxes (27), selection was performed to improve tameness and reduce anxiety and aggression. The clear selection response can be considered evidence that anxiety has a high realized heritability and shows that it is possible to reduce anxious temperaments through breeding. In an open-field study

(4), mice were categorized as fearful or not fearful based on their activity level and three selection lines (fearful, not fearful, and controls) were established. A strong selection response was shown both in the fearful and the not-fearful line. Another study demonstrated strong selection response when breeding for either anxious or outgoing temperaments in English pointers (18). The researchers indicated that the anxiety was a result of additive gene action.

A number of neurotransmitters like dopamine, adrenaline, noradrenaline, serotonin, acetylcholine, glutamate and monoamine oxidase are important neurotransmitters in the brain and are known to influence behaviour 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 into the synapsis and binds to the neurotransmitter receptors at the next neuron.

Regulation of the amount, release and reuptake/termination of these neurotransmitters is crucial for optimal neurological and mental function. Each neurotransmitter is regulated by mechanisms including high numbers of different receptors, transporters and reuptake systems that work together in complex interaction. Each of the receptors are encoded by specific genes, and different genetic variant in these genes influence the function of the receptor and "success of" neurotransmission in the synapsis.

Research has shown that dopamine levels in the amygdala can influence general anxiety level in human (10). Another study in humans found that low dopamine reuptake was associated with increased anxiety and irritability (12). Genes related to dopamine regulation and function have also been associated with anxiety and behavioural issues in dogs (15) (16) (17). 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 (24). A polymorphism in the 3'UTR-region of the human gene has been found to be associated with dopamine receptor density and anxiety, in close interaction with the dopamine transporter gene DAT (11).

Extensive research is performed in human psychiatry and animal models to understand the molecular influence of psychiatric disorders, including various forms of anxiety. The identification of associations of specific behaviour phenotypes to genetic variants of candidate genes would be an important step in an increased understanding of etiology and would be invaluable to improve medication through better psychopharmaca. For dogs, such information would be a valuable tool in selection for more robust individuals aiming at an improved animal welfare. The main goal of this study was to look for potential associations between the DRD2 gene and anxiety in several breeds where either generalized anxiety or noise sensitivity occur frequently.

Materials and methods

Dogs

A candidate study on DRD2 and generalized anxiety in Havanese was conducted as part of a master thesis. Because studies have found that different types of fear related behavioural disorders commonly co-occur (19) (26), the SNPs that were significantly associated with general anxiety in Havanese were later investigated for possible association with noise sensitivity in 5 breeds.

Data on general anxiety in Havanese and noise sensitivity in 5 breeds (including Havanese), was collected from privately owned dogs in collaboration with breed clubs and owners (Table 1). A general request was sent to all members of the breed and dogs were selected independently of behaviour, based on owners who responded and allowed DNA-sampling.

[Table 1]

EDTA-blood samples were collected from all dogs with owner's consent by certified veterinarians, in agreement with the provisions enforced by the Norwegian Animal Research Authority. Genomic DNA was extracted using E.Z.N.A blood DNA kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's recommendations and subsequently stored at -20 degrees celsius. The samples were collected (according to rules for ethical approval for collecting blood samples, FOR-2010-07-08-1085, FOR-1996-01-15-23, Regulation on Animal Experimentation). Performagene™ buccal swabs (DNA Genotek Inc) were used when blood sampling was impossible due to geographic distance. DNA was extracted following the manufacturer's recommendations.

Behavioural classification

Generalized anxiety

General anxiety in the Havanese was classified through an observational evaluation (performed by KB) and through a behavioural questionnaire (owner classification).

Observational evaluation

At the time of DNA-sampling, the dogs were observed and classified for three criteria (contact seeking, tail position and reaction to gentle restraint). Dogs that showed signs of anxiety in all criteria (table 2) were classified as cases and dogs free of symptoms in all criteria were classified as controls. A total of 104 Havanese obtained an observational classification. Out of these, 28 dogs were classified as cases and 33 were classified as controls.

[Table 2]

Owner questionnaire

The questionnaire consisted of 9 questions concerning anxiety related traits (the questions are listed in supplementary materials). The owners were asked to agree or disagree with various statements on the dogs' behaviour. Answers were given on a five-point scale, indicating high or low levels of anxiety. The average of all answers were then calculated to indicate the individuals' general anxiety level (owner score)(figure 1).

Owners of 150 dogs responded to the questionnaire. Dogs with more than 2 missing answers were excluded. The lowest recorded individual owner score was 1.22 and the highest was 5.0. The average score was 4.12. Cut-off for cases was set 0.5 σ below average owner score (OS) and the cut-off for controls was set 0.5 σ above average OS. 43 dogs were classified as cases and 60 dogs were classified as controls.

[Figure 1]

Noise sensitivity

Regarding noise sensitivity in the breeds Collie, Irish softcoated wheaten terrier (ISWT), Nova scotia duck tolling retriever (NSDR), Standard poodle and Havanese (table 3), owners answered 4 questions concerning reactions to loud noises including gunshots, fireworks, thunderstorms and heavy traffic. Answers were given on a five-point scale, indicating high or low levels of noise sensitivity. Cases were defined as dogs with a score of ≤2 in at least one of the 4 categories and controls had a score of ≥4 in all categories.

[Table 3]

Selection of candidate genes

Because the phenotype of interest in the Havanese was a generalized form of anxiety, we searched for candidate genes associated with general anxiety disorder (GAD) in humans. A thorough literature study (13) revealed a number of potential candidate genes associated with generalized anxiety. DRD2 (Dopamine receptor 2) has been shown to be associated with anxiety in people (11) and was chosen as the primary candidate gene for this study.

Primers

Primers embracing all exons and UTRs were designed based on the reference dog genome (CanFam3.1), using Primer3plus. Amplification was successful for all parts except for exon 1 and parts of the 3'UTR. Optimal temperatures were detected using a temperature gradient PCR-program with temperatures ranging from 54 to 64 degrees Celsius.

Sequencing of the PCR products were performed following a standard Sanger method on an ABI 3500 XL DNA analyzer (Applied Biosystems, Life Technologies of Thermo Fisher Scientific), followed by manual inspection using the Sequencher software from Gene Codes Corporations.

Primers and optimal temperatures are listed in the supplementary table 1.

Results

Detection of SNPs

All exons were initially sequenced for a small group of eight unrelated Havanese to identify regions with variation. Eight SNPs (table 4) were identified in the DRD2 gene; three located in introns, three located in exons and two located in the 3'UTR. The three exonic SNPs were synonymous.

[Table 4]

Exon 2

The two synonymous SNPs in exon 2 showed relatively balanced allele frequencies and were evaluated for association to anxiety related traits. First, generalized anxiety in Havanese and later noise sensitivity in the breeds Collie, ISWT, NSDR, Standard poodle and Havanese. The allele frequencies varied between the breeds and can be found in table 5.

[Table 5]

Observational classification

Significant association was detected between the two SNPs in exon 2 of the DRD2 gene and observed level of anxiety in the Havanese. In the first SNP (5:19782667), the allelic odds ratio was 4.35 (P-value 0.0008) (T = beneficial allele). In the second SNP (5:19782829), the allelic odds ratio was 4.07 (P-value 0.0010) (C = beneficial allele).

Owner Score

Significant association was detected between general anxiety (owner score) and the first SNP (5:19782667) with allelic odds ratio of 1.96 (P-value 0.0283) and the second SNP (5:19782829) with allelic odds ratio of 2.22 (P-value 0.0095). The average behavioural score of each genotype can be found in table 6.

[Table 6]

Noise sensitivity

Significant association between noise sensitivity and SNPs in exon 2 of the DRD2 gene was detected for ISWT and Collie. Significant association was found between the first SNP (5:19782667) and noise sensitivity in the ISWT, with allelic odds ratio of 2.64 (P-value 0.0371) (T = beneficial allele). Association between noise sensitivity and the second SNP (5:19782829) was significant in the ISWT with allelic odds ratio of 2.88 (P-value 0.0227) and in the Collie with allelic odds ratio of 3.03 (P-value 0.0319) (C = beneficial allele in both breeds).

Discussion

A significant association was detected between generalized anxiety in the Havanese and two SNPs in exon 2 of the DRD2 gene, for both owner score and evaluation by an external evaluator. A significant association was also detected between the first SNP (5:19782667) and noise sensitivity in the ISWT (this SNP showed no variation in the Collie breed) and between the second SNP (5:19782829) and noise sensitivity in the ISWT and Collie. Because the SNPs are synonymous, the functional effect associated with the SNPs are most likely due to the effect of variation in linked sequences/modifications. We found no association to noise sensitivity in the Havanese, but the observed prevalence of noise sensitivity was relatively low in the Havanese breed compared to the other breeds.

Generalized anxiety in the Havanese was classified through both an owner questionnaire and observation by an external evaluator. Observations made by the owner and observations made by an external evaluator have different strengths and weaknesses (23). The major weakness of owner evaluation may be that owners will evaluate dogs differently based on their skills and frame of reference. The evaluation may also not be completely objective since some owners may be reluctant to classify their dog as anxious.

Previous studies have shown that complex behavioural patterns in dogs can be reliably evaluated by an experienced person and that a few well selected characteristics may be sufficient in order to describe the differences between dogs (30).

Subjective bias in owner evaluation (e.g. systematic under-reporting of anxiety) could be a challenge if an owner/breeder was reporting several dogs from a certain line/genotype, which could lead to false association. The number of Havanese per owner in this study was 2.08, and therefore we do not believe that the owner classification represents a systematic problem. Most owners did however rate their dogs quite high, indicating low levels of anxiety.

In the owner-based classification, we observed that the criteria/threshold for inclusion of dogs as cases/controls could have a marked influence on the results. This demonstrates the challenge of a biologically correct behavioural phenotyping and may, together with genetic heterogeneity, explain the variable reproducibility of many studies on genetics of behaviour. To reduce the frequency of misclassification and obtain a clear difference between cases and controls, cut-offs were set 0.5 σ above and below average owner score. We also chose a narrow inclusion criteria for the controls to correct for a potentially too mild evaluation by the owner. Because of the quantitative nature of anxiety traits and the numerous genes involved, strict inclusion criteria for cases and controls would increase contrasts and increase the likelihood of identifying protective alleles and detecting association between the gene and the trait.

We cannot rule out the possibility that owners of anxious dogs are either more or less inclined to participate in a study like this, which would cause some deviation between the true and observed prevalence of anxiety. However, this would probably not affect genotype-phenotype association, or the results of this study.

Conclusion

SNPs in exon 2 of the DRD2 gene are significantly associated with generalized anxiety in the Havanese and noise sensitivity in Irish softcoated wheaten terriers and Collies. The same alleles were beneficial in the three breeds. There was no significant association between noise sensitivity and the SNPs in the Havanese, Nova scotia duck tolling retriever or Standard poodle. Because the SNPs are synonymous, the functional effect associated with the SNPs is most likely due to linked mutations.

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Tables and figures

Table 1: Breeds and number of dogs included in the study

Breed (abbreviation)	Number of dogs (females, males)
Havanese	158 (92, 66)*
Collie	94 (62, 32)
Irish softcoated wheaten terrier (ISWT)	44 (27, 17)
Nova Scotia duck tolling retriever (NSDR)	33 (17, 16)
Standard poodle	29 (19, 10)
Total	358 (217, 141)

*total number of Havanese that participated in at least one of the studies (observational, owner score and/or noise sensitivity)

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

Table 3: Breed and number of cases and controls, noise sensitivity

Breed (abbreviation)	Number of dogs (cases, controls)
Havanese	121 (25, 96)
Collie	94 (49, 45)
Irish softcoated wheaten terrier (ISWT)	44 (20, 24)
Nova Scotia duck tolling retriever (NSDR)	33 (16, 17)
Standard poodle	29 (15, 14)

Table 4: SNPs identified in the DRD2 gene (Havanese)

Index	Intron/exon	Location (CanFam 3.1)	Alleles (CanFam3.1 in bold)	Amino acid change
1	Intron 1	5:19782497	**g**/a	-
2	Exon 2	5:19782667	**c**/t	Synonymous
3	Exon 2	5:19782829	**t**/c	Synonymous
4	Intron 4	5:19787766	**t**/c	-
5	Intron 4	5:19787788	**c**/t	-
6	Exon 7	5:19791794	**c**/t	Synonymous
7	Exon 8, 3'UTR	5:19794262	**a**/g	-
8	Exon 8, 3'UTR	5:19794287	**t**/c	-

Table 5: Allele frequencies (%) of the SNPs in exon 2 in the DRD2 gene

	Alleles	Havanese	Collie	ISWT	NSDR	Standard poodle
5:19782667	C/T	64/36	0/100	61/39	55/45	36/64
5:19782829	T/C	63/37	13/87	60/40	50/50	48/52

Table 6: The average behavioural score of each genotype

SNP 1 (beneficial allele in bold)	Average OS*	SNP 2 (beneficial allele in bold)	Average OS*
CC	4.00	TT	3.94
CT	4.15	**T**C	4.18
TT	4.39	**CC**	4.42

*OS=Owner score

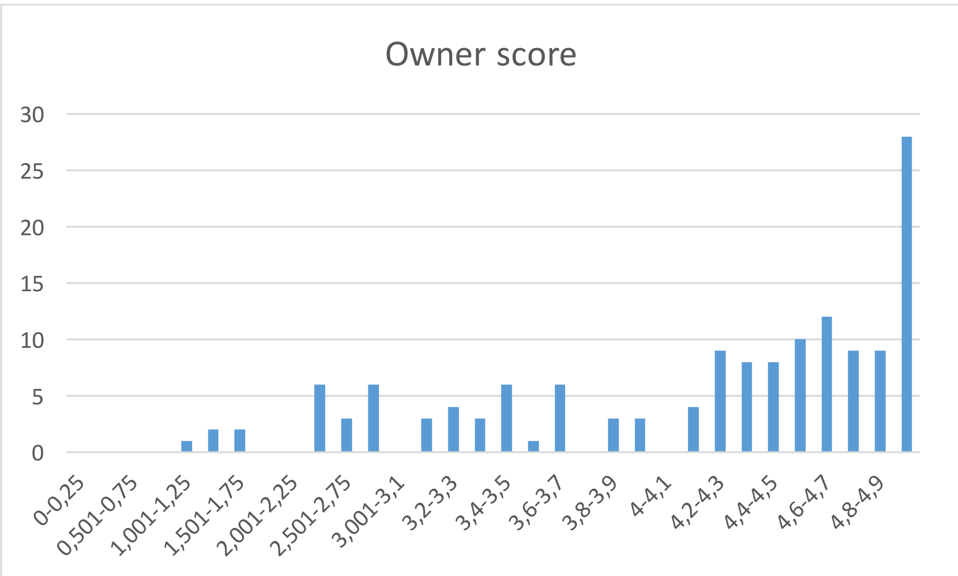

Figure 1: Distribution of average owner scores

Supplementary materials

Supplementary table 1: Primers

Exon	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	GCCCGTAGCACCCAATCTT	TAGCACTACCCCGGCAGAT	58
8	CGGACCAGGCCTTCTCTTTG	CTTCTCTGGGGTTCAGCCTG	No product
8	GGTGGGGATGGACAGTTCAC	AGTGGTTTTGTGGCAGGAGG	62
8	TCGTAGCAATTGTTGGGCCT	GGGTCACCCTTTCTTGGAGG	No product

Supplementary table 2: Questions used for owner questionnaire

Questions - havanese

1. Is your dog scared of other dogs?
2. Is your dog scared of strangers (children)?
3. Is your dog scared of strangers (women)?
4. Is your dog scared of strangers (men)?
5. Is your dog scared of people with unusual behaviour?
6. Will your dog pull away from other dogs on a walk?
7. Will your dog pull away from strangers who want to pet him/her on a walk?
8. Would you say your dog is jumpy?
9. Would you say your dog is a wuss?

"Strongly agree" was considered a 1 and "strongly disagree" was considered a 5.