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Genetic variation in candidate genes of behaviour and associations with behaviour phenotypes in dogs

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Introduction

Domestication

Domestication of the dog (Canis lupus familiaris) dates back at least 14 500 years ago to the Palaeolithic era as evidenced by a canine jaw bone found at the Bonn-Oberkassel archaeological site in Germany(Benecke, 1987). However, domestication studies based on the canine genome have provided nuance and debate concerning the timeline of domestication. One study on dogs in Ireland found two deeply split heritages suggesting two separate domestication events: One in Europe and one in Asia. This supported one leading theory of dual domestication, in which dogs were domesticated on two separate occasions and the Asian dogs were later introduced into European populations (Frantz et al., 2016). Other studies based on calculating the rates of mutation in canine haplotypes show evidence for only one domestication event 20-40000 years ago(Botigué et al., 2017). Results from studies on domestication provide evidence that the dog ancestors split into two populations; Asian and European, but it's unclear when this divergence occurred and whether dogs were domesticated once or twice.

Domestication led to changes in behavioural traits and morphological traits, such as coat colour, tail shape, and body size. Physiological traits such as the ability to better digest starches(Axelsson et al., 2013) and behavioural traits such as tameness and sociability. A concordant selection for behaviour and changes in morphology is explored in the well-known Russian Farm fox experiment, where caged un-tamed foxes were selected purely for tameness, and after 8-10 generations, the more domesticated foxes started showing changes in coat colour, rolled tails and higher prevalence and degree of floppy ears compared to their wild cousins.(Trut, 1999). Whether these morphological traits in silver foxes are either incidentally selected because of inbreeding, or because they are associated with loci associated with behaviour, it would either way be expected that mutations or new structural variants associated with behaviour, might have higher fitness in a general domestication process for all animals. Recent behaviour studies have investigated the relationship between canine behaviour and Williams-Beuren syndrome(WBS). WBS is a congenital disorder causing among other symptoms, hyper sociability in humans due to a deletion in chromosome 7. They found evidence for structural variations in the WBS locus in dogs contributed to extreme sociability in dogs. Phenotypes were classified using various puzzle solving, human-interaction and proximity-seeking tests. This may have had a positive impact on the domestication process in some populations. (vonHoldt et al., 2017)

Impact of maladaptive behaviour

Maladaptive behaviour in humans has a negative impact on welfare in those afflicted. It's likely that this is similarly true for dogs as well .Fear and anxiety are distressing mental states in and of themselves, but there are also other - more long term - negative side effects: Chronic anxiety and stress leads to increased cortisol levels, which predisposes to a host of diseases and comorbidities(McEwen, 1998). It likely also affects the dog-owner relationship in a negative way. Owners acquire pets for a number of reasons, the most common being companionship (Staats et al., 2008). However, it's been showed that owners less satisfied with their pets' behaviour also report weaker attachments to their pets(Serpell, 1996).

Behavioural problems are a common reason for relinquishment or euthanasia of adult dogs(Marston et al., 2004; Mondelli et al., 2004; Proschowsky et al., 2003; Salman et al., 1998). This is not only a welfare problem for companion animals, but one for the owners as well seeing as relinquishing pets is, perhaps not surprisingly, shown to be stressful (Marston et al., 2004). Dogs with behaviour problems like overt aggression, may also pose a public health risk. Dogs exhibiting aggressive

behaviour have a higher incidence of biting other dogs and humans (Guy et al., 2001) (Wright, 1991) causing various levels of pain and injury (pain and a need for antibiotics and medical care).

There is also evidence supporting comorbidity in some maladaptive) behaviours. For instance, fearful dogs have been shown to have higher levels of aggression, noise sensitivity and separation anxiety, compared to non-fearful dogs, illustrating the need for further understanding of canine behaviour and the underlying mechanisms.(Tiira et al., 2016)

Heritability of behaviour traits - twin and adoption designs

The nature vs nurture debate predates modern science. In John Locke's *An essay concerning human understanding* (Locke, 1689) he criticizes Descartes' idea of innatism; that humans are born with innate ideas and knowledge. Locke argued that humans were born as a blank slate at birth. This debate was, at the time confined, to religious and philosophical opinion, and had little to do with science.

The modern interpretation of the phrase was coined by Francis Galton, who is oft credited as the originator of the science of behavioural genetics. This accreditation is based on his ground-breaking work "Hereditary genius - an inquiry into its laws and consequences" (Galton, 1869), in which he described the first statistical investigations of whether personality traits are hereditary.

The nature vs nurture debate has been proven to be a false dichotomy and has been replaced by a complex understanding of an interplay between environmental effects and genes. Behavioural studies are not investigating whether a trait is genetic or not, but rather the proportion of genetic influence on phenotype, also known as heritability.(Plomin et al., 2012)

Heritability is the proportion of phenotypic differences among individuals that can be attributed to additive genetic differences within a defined population. Typically described either as a percentage or fraction of 1, where 0 meaning none of the differences is attributed to genetics, and 1 meaning all the differences are explained by genetics.

Psychiatric disorders

Human behavioural genetics have had much focus on the heritability and inheritance of psychopathologies. A major reason for this focus, is the high prevalence and high impact on Quality-of-life (QoL) of this group of diseases. (Steel et al., 2014) This furthers the need to gain a deeper understanding of the aetiology of these diseases. Psychopathologies that are studied are usually based on accepted clinical definitions in the diagnostic manual of mental disorders (also known as DSM-V)(APA, 2013) or in the International Classification of Diseases and Related Health Problems (ICD-10) . These internationally accepted diagnostic guidelines facilitate research into this field. A consensus on diagnostic criteria of psychopathologic diagnoses, contribute to less variation due to classification/psychiatrist and makes it easier to identify the influence of genes. There is reported evidence of moderate to high heritability for numerous psychological disorders:

Schizophrenia

Schizophrenia has been a highly studied subject in the fields of psychopathology and behavioural genomics. This is likely due to it being one of the most severe psychopathologies (Solanki et al., 2008) in addition to being quite prevalent: Schizophrenia is defined as having delusions, hallucinations, disorganized thinking (speech), grossly disorganized or abnormal motor behaviour (including catatonia), and negative symptoms such as diminished emotional expression(Tandon et al., 2013). There are also a number of symptoms associated with schizophrenia like inappropriate affect, disturbed sleep, anxiety and phobias, depersonalization, derealization etc. which are not a part of the

diagnosis of Schizophrenia but are highly concurrent. About 1% of the population is reported to suffer from schizophrenia at one time in their lives (Saha et al., 2005).

Schizophrenia has consistently been shown to be familiar. The median risk of developing schizophrenia is 6% for parents of schizophrenics, 9% for siblings and 13% for offspring if one parent is affected. If both parents have schizophrenia the risk for offspring developing is 46% (Ritsner & Gottesman, 2011) The reasons why offspring of single-parents with schizophrenia have a lower morbidity risk than parents of , are not investigated. One likely aspect is the relatively early age of onset for Schizophrenia. It is usually diagnosed at early adulthood, meaning that if an at-risk individual has reached child-rearing age, and that offspring has reached adulthood, it is statistically less likely they will be diagnosed with Schizophrenia.

In 14 reared-apart twins, where at least one twin had schizophrenia, 9 of the twin pairs were both schizophrenic (64%) (Gottesman, 1990) and offspring of affected persons or non-affected persons with an affected twin strongly increases the risk of disease (McGuffin, P. et al., 1987). The explanation being that non-clinical twins did not develop disease due to environmental effects, but transferred their genetic disposition to their offspring who then turned out to have the same risk as the offspring of affected individuals(Gottesman & Bertelsen, 1989). A study using data on family relationship and schizophrenia prevalence in Taiwan estimated a heritability of about 47%. Having an affected twin was associated with a relative risk of 37.86 (Chou et al., 2017). Other meta-analysis of twin studies found heritability in liability to schizophrenia to be 81% (Sullivan et al., 2003)

Anxiety

In human medicine anxiety disorders cover a wide range of phenotypes with quite dissimilar clinical presentation which all involve anxiety in some way. Panic disorder (PD) involve sudden panic attacks lasting for minutes. This is contrasted by General anxiety disorder (GAD) which is a more chronic state of diffuse anxiety. Anxiety disorders (AD), while not as severely detrimental to quality of life as schizophrenia, is the most common group of mental disorders with a lifetime prevalence of 29% (Kessler et al., 2005). Anxiety has a significant impact on QoL for millions of people, affects QoL of family and is a costly disease for the public health system. This group of disorders increase the risk of unemployment and suicide (Katzelnick et al., 2001; Senaratne et al., 2010).

Most anxiety disorders are influenced by genetics, depending on type of AD in question. A Meta-analysis based on twin and adoption studies have shown estimates of 32% and 43% heritability for GAD and PD respectively (Hettema et al., 2001).

Twin studies on individuals not previously diagnosed with PD, showed a liability towards PD with an average heritability of 38%. The phenotypes were based on phone questionnaires and an algorithm matched answers to the DSM-IV (Mosing et al., 2009). Another large-scale twin study, investigating the heritability and environmental effects on several DSM-IV disorders found a heritability of 30% for PD. They also found that the liability towards all disorders was more heritable (54%) than each individual disorder (ranging 28%-40%)(Tambs et al., 2009). In this study generalised anxiety showed moderate heritability of 26%. Earlier studies on general anxiety have found no heritability. (Andrews et al., 1990)

Substance dependency

A meta-study of 12 twin and 5 adoption studies on alcohol use disorder estimated a heritability of about 50%(Verhulst et al., 2015), and another meta-study of 50 family, twin and adoption studies, found a weighted average heritability of 12%, with an upper limit of 36%(Walters, 2002)

Mood disorders

Mood disorders are a diverse group of psychologic disorders, with serious and significant impact on quality of life. The lifetime risk of suicide for people diagnosed with mood disorders are estimated at 19%(Goodwin & Jamison, 1990). The two major categories of mood disorders are Major Depressive Disorder (MDD) and Bipolar Disorder (BD). Both categories having episodes of severe depression, but bipolar disorder also displays episodes of mania or euphoria. (APA, 2013). The prevalence of MDD is high, with a lifetime risk of about 17%, affecting adult women at twice the rate of adult men. BD is less common, with an incidence of about 4% among adults. (Kessler, 2008)

The heritability of mood disorders has been extensively investigated. Reviews have shown a higher first-degree family risk for MDD and BD, compared to controls (9% vs 3% for MDD and 9% vs 1% for BD). There is evidence that BD might be a more severe form of MDD, seeing as relatives of BD-cases have a higher risk of MDD, but the reverse is not true (McGuffin, Peter et al., 1987). However, there is also evidence from twin-studies that do not support this (McGuffin et al., 2003). The relationship, or lack thereof, between MDD and BD, is yet to be understood. Identifying the genes influencing phenotype will provide a crucial understanding of the disease mechanism.

Twin studies have shown a high degree of genetic influence on mood disorders. Reviews on twin studies have shown an average twin probandwise MDD concordance of 0.43 for MZ twins and 0.28 for DZ twins, and heritability of 37% (Sullivan et al., 2000). Later investigations have found similar results, and an average heritability of 38% (Kendler et al., 2006). However, there is evidence that more severe depression is also more heritable. One study on clinically ascertained MDD showed the heritability of MDD-diagnosis to be 70% based on clinical diagnosis of zygosity-blinded twins.(McGuffin et al., 1996).

So far, investigations into which genes are influencing the phenotype have provided varied evidence, but there is strong and replicated evidence that there is a link between MDD and 15q, and BD and the 13q and 22q regions. (Plomin et al., 2012)

Personality traits

One of the most studied psychological traits, apart from psychopathology, is intelligence. Intelligence is a behavioural trait that can be easily phenotyped and quantified based on commonly accepted intelligence tests (Boake, 2002). Several meta-analysis studies have been conducted and are showing about 50% heritability for general intelligence (Chipuer et al., 1990; Haworth et al., 2010).

In contrast to psychopathologies, personality phenotypes have usually been identified based on self-report questionnaires (Davis, 1999). After decades of research, personality-research identified a taxonomy of 5 major personality dimensions, now commonly referred to as “the big five” in personality. The big five are 1) openness to experience, 2) conscientiousness, 3) extraversion, 4) agreeableness and 5) neuroticism (Gosling et al., 2003).

Personality traits in general have consistently shown about 30-50% heritability in human twin and adoption studies over the last 50 years (Power & Pluess, 2015a) Five major domains of individual differences in human behaviour: 1)Cognitive abilities, 2)personality, 3)social attitudes, 4)psychological interests, and 5)psychopathology e.g. Autism Spectrum Disorder (Bouchard & McGue, 2003; Jang et al., 1996), Schizophrenia and Anxiety Sensitivity (Stein et al., 1999), have been estimated to have approximately 40-50% heritability. There is also evidence of a high degree of political voting heritability (Fowler et al., 2008).

The heritability of behavioural traits has been consistently replicated and evidenced in the late 21st century and In 2000 Erik Turkheimer concluded based on empirical research in the last century that "All human behavioural traits are heritable" (Turkheimer, 2000).

Limitations of twin studies

Twin studies are a common method for estimating heritability and genetic influence. The use of twin studies can, however, be biased. For instance, a more equal environment is expected among twins. It would be expected that MZ twins have more similar environment than DZ twins (Kendler & Baker, 2007), and their prenatal environment is also more similar. (In some cases, adoption studies where, twin pairs are adopted by different new parents a short time after birth, have been compared to twins growing up together to get an estimate of the effect of common environment.)

Dogs, hunting traits, aggression, anxiety and function tests

Early studies into canine behavioural genetics were investigating breed behaviour. Scott and Fuller demonstrated interbreed differences between Cocker Spaniels, Beagles, Basenji, wire-haired fox terriers and Shetland Sheepdogs. They were all reared in a standardized manner but showed differences in trainability, reactivity, problem-solving abilities and a number of other phenotypic axes (Scott & Fuller, 1974). This represented early empirical evidence for genetic components of canine behaviour.

Heritability of various behaviours in dogs has shown a high variation concerning the genetic components. Wilsson and Sundgren found medium-high (20-53%) heritability for 10 traits studied (Wilsson & Sundgren, 1998) with some traits showing a very high heritability. Another study found the heritability of some traits to be small (Reuterwall & Ryman, 1973). When comparing cat littermates, and their tendency for different behaviours; Turner et al found a paternal influence on sociability and hostility (Turner et al., 1986). A different study found similar results where kittens sired by friendly phenotype toms were more approachable to people and bold towards novel situations (McCune, 1995).

Canine aggression studies have, until recently, not been able to identify specific genes involved in aggression. Van den Berg studied general unspecific aggression in golden retrievers and initially found no clear evidence for a genetic component in aggression. However, they formulated the hypothesis that the dogs displayed several heterogenous aggressions, meaning there might be different types of aggression, e.g. a difference between human-related aggression and dog-related aggression. This implied there could be different genetic pathways contributing to different sorts of aggression, which could be the reason they initially found conflicting evidence for heritability (van den Berg et al., 2003). Linamo et al used CBARQ questionnaires for phenotyping Golden retrievers and estimated heritability of aggression towards humans and dogs as 0.77 (S.E. 0.09) and 0.81 (S.E. 0.09) respectively (Liinamo et al., 2007). Duffy et al demonstrated, through the use of internet-based questionnaires, the presence of subtypes of aggression towards owners, dogs and unfamiliar humans, and that these subtypes varied considerably from breed to breed with small breeds showing greater prevalence of human-directed aggression (Duffy et al., 2008). Våge et al investigated human-directed aggression in a varied-breed population of dogs and found haplotypes significantly associated with aggressive phenotypes, in Dopamine receptor gene D1 (DRD1), Serotonin-receptor gene 1D (HTR1D), Serotonin-receptor gene 2C (HTR2C) and solute carrier family 6 (neurotransmitter transporter, gamma-aminobutyric acid) member 1 (SLC6A1).

Table 1.

Brenøe et al (Brenøe et al., 2002)investigated hunting behaviour in three breeds of gun hunting dogs in Norway; German Short Haired Pointer, Wire-haired pointer (Wire-Haired) and Brittany Spaniel (Breton). Using individual hunting ability tests for 7 different traits; (hunting eagerness, speed, style, independence, seeking width, ability to work in the field and cooperation) they found a high degree of genetic correlation between these different hunting traits. The heritability (h^2) was found to be between 6% to 28% for the different breeds. *(Table 1)*

H^2 estimates for 7 hunting-traits

	Short-Haired	Wire-Haired	Breton
Ability to work in the field	0.25	0.18	0.20
Bird-finder index	0.04	0.05	0.03
Cooperation	0.21	0.10	0.09
Hunting eagerness	0.28	0.17	0.19
Independence	0.14	0.21	0.06
Seeking width	0.25	0.17	0.21
Speed	0.26	0.18	0.23
Style	0.27	0.16	0.20

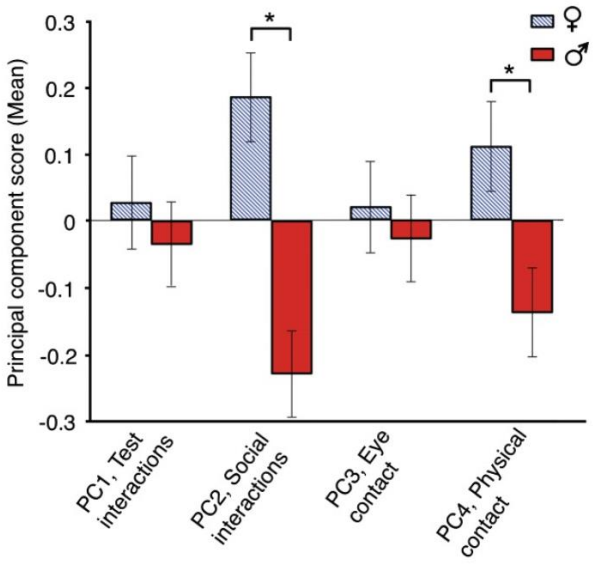
A behaviour study utilizing almost 500 research beagles found that during an unsolvable task, the degree of interaction with behavioural test-apparatus and social interaction with humans had an estimated heritability of 32% and 23%, respectively. The degree of Interaction with apparatus was measured as the product of frequency and duration the dog would interact with the unsolvable task (In this case immovable plexiglass with a treat under). The degree of Social interaction with humans during the unsolvable task was measured as the product of frequency and duration of eye contact and physical

contact with one tester who was present in the room. Two other principal components of the test, eye contact and physical contact with the tester, had low degrees of heritability (Persson et al., 2015) *(Table 2).* They also found that bitches had higher degrees of social human interaction and physical contact compared to males. *(Figure 1)*

Table 2

Principal components	h^2
Test interactions	0.32
Social interactions	0.23
Eye contact	0.0008
Physical contact	0.0005

Figure 1



Reviews on canine behavioural heritability generally find low levels of heritability for personality traits (Hall & Wynne, 2012; Houpt, 2007; Mackenzie et al., 1986). It's important to note a limitation on this kind of narrative reviews, is the inherent subjective bias, present in all reviews not using an objective statistical approach to analysis i.e. meta-analyses or systematic reviews.

A meta-analysis of 47 behaviour studies, categorized all the different personality traits into five major categories: Hunting, Environment, Play, Herding and Physical Characteristics. They complied with earlier reviews and found low general heritability for behavioural traits (Hradecká et al., 2015) (Table 3). It should be noted that the arbitrariness of the categories, and which traits were included, was only briefly touched upon and not discussed in the paper. The categorization of traits will undoubtedly have a significant impact on the heritability estimates.

Table 3. Heritability, confidence interval, number of heritability coefficients entering the analysis, and total sample size for the categories Environment, Herding, Hunting, Play and Psychical characteristics.

Category	**Heritability**	**Lower limit**	**Upper limit**	***p*-Value**	***n* heritability coefficients**	**Sample size**

Environment	0.154	0.141	0.166	0.000	119	197,258
Herding	0.099	0.067	0.132	0.000	99	26,175
Hunting	0.154	0.141	0.166	0.000	247	521,741
Play	0.093	0.057	0.130	0.000	2	2,811
Psychical characteristic	0.123	0.114	0.132	0.000	1232	1,890,613

Interpreting heritability

Heritability estimates vary depending on study design and population (Turkheimer et al., 2003). Fontaine et al found that for children divided into 4 groups of developmental trajectories in relation to callousness-unemotional traits (stable low, stable high, declining, rising) had a different heritability of membership to the groups depending on sex. When estimating the heritability of belonging to a certain group of developmental emotional trajectories, the girls had a different degree of heritability than boys. For boys, 60-80% of the liability for group membership was attributed to genetic influences, but genetic influences with girls were relatively weaker and environmental influences relatively stronger. In essence, he found that there were sex differences between the heritability of developing certain emotional traits (Fontaine et al., 2010)

Since heritability is a proportion between genetic variance compared to total phenotypic variance (including environmental variance) the heritability is strongly affected by environmental factors. If all environmental influences are fully identical, heritability will theoretically be 100%, meaning that only the genome influences phenotype. There are empirical examples that there are environmental factors that strongly influence the estimated heritability for some traits. For instance, alcohol and tobacco use show a higher heritability under conditions that facilitate substance use e.g. during low taxation on substance (Boardman, 2009), readily available alcohol (Kendler et al., 2011) or social norms encourage drinking (Kendler et al., 2011). In other words, when these environmental conditions are present, genetic influence on phenotype (alcohol use) is enhanced, showing the complex relationship between environment and genetics.

Even traits with the highest degrees of heritability, can be strongly influenced by environment in special situations. Height has shown to have a very high degree of heritability, in many cases up to 90%. Yet North- and South-Koreans, sharing the same genetic background, has an average height difference of 6-13 cm (Pak, 2004; Schwekendiek, 2009). This illustrates the importance of remembering that a heritability estimate is specific for a specific population at a specific time with its associated genetic and environmental variance and may not be valid in other populations for the same traits. Heritability of height is also high in dogs, but within each breed, the heritability is low, due to a low additive genetic variance for height within a breed.

Neurophysiological impact on behaviour

Since discovering genetic influence on behaviour, significant research efforts have focused on finding the neurophysiological mechanisms involved. Much of this research has investigated the pathways of neuro-signalling in the brain (Hanin, 1978) (Mesulam, 2000). Neurons in the brain connect to each other and communicate through multiple branches and form upwards of a trillion connections. Interneural signal transmission between all neurons pass via a chemical synapse, where each electrical impulse in a signal is translated into a chemical signal, then translated back into an electrical signal: An electric action potential travels down the axon, reaches the Voltage-gated Calcium-channel, which open and results in an influx of Ca^{2+}. This triggers the exocytosis of vesicles containing variable effector proteins i.e. neurotransmitters. Neurotransmitters are molecules able to bind to neurotransmitter receptor-proteins on the postsynaptic membrane and triggers an activation or inhibition of the target cell. (Figure 2)

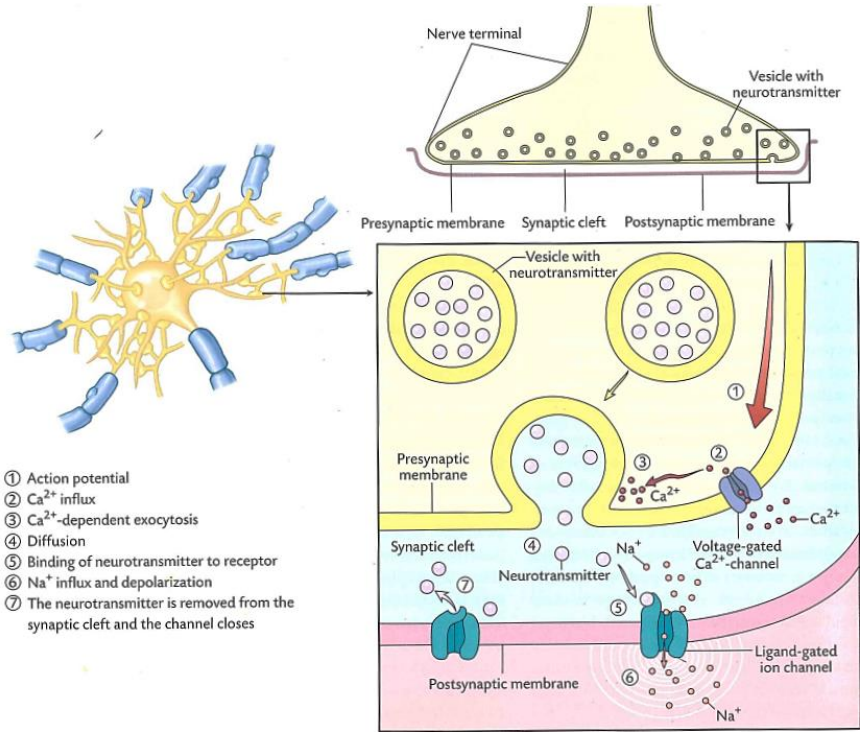


Figure 2 - Sjaastad, Sand & Hove, 2010 2e

In the synapses, multiple gene products regulate signal transduction in a complex network of protein interactions. Separate genes produce numerous receptors, transmitter-transporters, transmitter degrading enzymes and numerous other proteins. There are also several receptors for the same transmitter, which may have opposite inhibitory vs excitatory reactions, in the target cell. It is also shown that a single neuron may release several different neurotransmitters (Trudeau, 2004). It's obvious that mental processes in the brain are dependent on these neurochemical pathways, but their specific impact on complex behaviour is near-impossible to predict. A significant amount of information has come from studying the clinical effects of natural and synthetic chemicals like nicotine, cocaine, lysergic acid and opioids (Valenstein, 2002).

In the last 50 years of medicine, major depressive disorder has been recognized as being linked to an imbalance in monoamine (Serotonin, dopamine, and noradrenaline) regulation in the central nervous system. This has been dubbed the monoamine hypothesis (Bunney et al., 1965; Delgado, 2000). The monoamine hypothesis is both the reason for, and corroborated by, monoamine targeted pharmacological therapies as a method of treatment, and later in the 1970s, serotonin specific therapies (Wong et al., 2005). However, simple depletion of monoamine levels probably does not lead to symptoms of depression in healthy humans (Salomon et al., 1997) or worsen symptoms in depressed humans (Berman et al., 2002). Because of efficacy and minimal side effects compared to other monoamine therapies, selective serotonin reuptake inhibitors (SSRI), have become the major drug for treating depression (Wong et al., 2005), generalized anxiety disorder (Kapczinski et al., 2003; Kasper, 2006; Patel et al., 2018) and obsessive-compulsive disorder (Soomro et al., 2008). Because of this, monoamine pathways have been candidates for several behavioural studies.

Peremans et al measured a higher density of Serotonin receptor 2A (HTR2A) in impulse aggressive dogs than in controls (Peremans et al., 2003). Tran et al found that low 5-HT concentration systemically or in the amygdala increased fear behaviour in rats (Tran et al., 2013). In humans, MAOA nonsense mutations lead to Brunner syndrome characterized by anti-social and violent behaviour. MAO-A knockout mice display higher levels of 5-HT, aggression and perseverative behaviour (Godar

et al., 2011), but MAO-A knockouts which were also treated with 5-HT synthesis showed reduced perseverative behaviour (Bortolato et al., 2013).

In a study on silver foxes, Popova et al demonstrated significant differences in serotonin metabolism enzymes and serotonin receptors between foxes selected for domestication and foxes selected for increased aggression (Popova et al., 1997)

A small-scale study on male shelter dogs (n=14), investigating association between 5-HT levels in blood and degree of sociability towards humans as defined by a battery of 7 sociability tests, found a weak linear, significant correlation between behavioural scores and 5-HT levels (Alberghina et al., 2017).

Riva et al found in a small scale study that anxious dogs have significantly higher plasma levels of dopamine and serotonin compared to controls (n cases/controls = 23/13) (Riva et al., 2008). The role of serotonin receptor 2A (HTR2A) in canine anxiety-disorder was investigated by Vermeire et al. They studied the HTR2A binding index in the brain regions related to human anxiety; frontal cortices and temporocorticals. They found that Binding index was significantly lower in anxious-disorder dogs vs controls, as phenotyped/diagnosed by behaviour-specialist veterinarians with supplementary phenotypes provided by owner questionnaires (C-BARQ)(Vermeire et al., 2009).

Phenotyping

Challenges in characterization/classification of behavioural traits

One challenge in behaviour research has been the accurate classification of behavioural phenotypes. Already in the 1940s the scientific community was aware of the challenges in measuring personality traits (Zeligs, 1942), which is illustrated in Leo Kanner's landmark case report of 11 children titled "Autistic disturbances of affective contact"(Kanner, 1943). In which Kanner not only recognized autism as a distinct syndrome but also the challenges related to quantitatively describing personality traits. He also noted the need for biomarkers as he saw a tendency towards autism in some families. As autism became more recognized in the 1970s, the concordance rate between monozygotic and dizygotic twins was reported as 36% and 0% respectively. The landmark study of Folstein and Rutter showed evidence that a more broadly defined phenotype, where one included language and cognitive impairments, had a markedly higher concordance (MZ = 82% vs. DZ = 10%) (Folstein & Rutter, 1977). This showed that autism, and potentially other disorders of behaviour, was not inherited in a simple Mendelian fashion, but rather as a complex disease with several genes contributing to the phenotype. This led to the finding of a cluster of linguistic, cognitive and social traits in family members of autistic persons, that paralleled the hallmarks of autism. These traits were milder but qualitatively similar to autism hallmarks. This led to the use of the Broad Autism Phenotype(Gerdts & Bernier, 2011) (Losh et al., 2008). The Broad Autism Phenotype (BAP) is a set of personality and language characteristics that reflect the phenotypic expression of (the genetic liability to) autism traits, in non-autistic relatives of autistic individuals. BAP is a cluster of so-called endophenotypes. These subclinical markers of disease are present both among the affected and the at-risk individuals, but the at-risk individuals are not affected strongly enough to be classified as having autism spectrum disorder according to the DSM-V. However they still represent genetic liability towards disease which is of interest for research, and is today normally measured using different questionnaires (Hurley et al., 2007).

The history of autism research parallels canine behaviour research, in that several behaviour phenotypes like anxiety, separation anxiety, and aggression, can be difficult to accurately phenotype and some clinical diagnoses e.g. separation anxiety, are very likely a broad phenotype. In the study of complex psychiatric disorders, e.g. autism or separation anxiety, endophenotypes (also known as

intermediate phenotypes) can possibly help overcome challenges in gene identification (Losh et al., 2008). Endophenotypes are heritable, co-segregate with the disease, i.e. broad phenotype (Autism, Anxiety etc), yet be present even when the disease is not (i.e. state independent), and can be found in non-affected family members at a higher rate than in the population. (Flint & Munafò, 2007)

Defining a phenotype in dogs is challenging. One major reason for this is the complex interaction between different behaviours. Since the definitions of most psychiatric diseases are based on descriptions of phenotypes, the aetiology has not traditionally been included in the diagnosis. It is likely that diseases which seemingly have a high degree of co-occurrence has a joint genetic liability towards disease.

For instance, several psychopathologies have a high degree of co-occurrence (Morisano et al., 2014). For instance, people suffering from depression have a higher risk of having other psychological diseases e.g. anxiety and substance abuse disorder (Hirschfeld, 2001). The likelihood that co-occurring psychopathologies have a joint genetic liability is significant, and there is evidence for this in dogs as well.

One questionnaire study on dogs showed a significant association between noise-sensitivity and general fearfulness (Storengen & Lingaas, 2015). Another study found corroborating evidence and also a co-occurrence with aggression (Tiira et al., 2016). Dogs with separation anxiety are also shown to have higher noise sensitivity (Overall et al., 2001).

Based on data from the Swedish Dog Mentality Assessment, Saetre et al found a high degree of correlation between different scores and identified Shyness-Boldness and Aggression as two generalized traits underlying many behavioural scores (Saetre et al., 2006), implicating there may be a smaller number of genes influencing a large number of observations.

Major challenges in phenotyping behaviour are the varied and broad phenotypes for many behaviours and the complex inheritance of these behaviours.

Currently, there are four general approaches to phenotyping behaviour in dogs.

1. Behaviour Tests (Wilsson & Sundgren, 1997) (van Rooy et al., 2014).

Tests that expose the subject to stimuli and measure its response. Some measure only on a single trait like the Ainsworths Strange situation test (Mary & Bell, 1970), others measure multiple traits. Examples include; puppy tests for working dogs (Seeing-eye dogs, mine detection dogs etc), and the Dog Mentality Assessment (DMA) developed for the Swedish kennel club (Curt Blixt, 2010).

In multiple traits testing the dogs are either in a room or guided through a course, where they are subjected to different stimuli. The responses are observed, usually with a video camera and graded in a quantifiable manner. For instance, the dogs are subjected to a loud noise, and its initial reaction is graded on a usually linear scale according to predetermined descriptions of the types of reactions that are expected. E.g. "*1= little/no reaction, the dog might look towards the noise but does not display signs of surprise, posture is confident*." While "*4=High reaction. The dog has a vocalizing response or tries to flee from noise and displays a fearful posture*."

Different tests have varying levels of detailed descriptions of the traits and grades with which they score the dogs. This kind of test has historically been viewed as the most objective and "the gold standard" which other methods have been compared to. It is also a common way of phenotyping behaviour in laboratory animals i.e. mice and rats (Henderson et al., 2004; Holmes et al., 2003; Scott & Fuller, 1974).

One limitation of interpreting behaviour testing is the lack of inter-test standardization for many parameters. E.g. Indoor vs outdoor, the choice of noise for startle-reaction or noise sensitivity, age at testing etc (Diederich & Giffroy, 2006). This affects the meta-analysis of behaviour tests, limiting their impact. The Swedish Dog mentality assessment has been validated, using owner questionnaires (C-BARQ, see below) to show similar results for the same dogs (Svartberg, 2005). (

2. Owner directed survey/questionnaire. Questionnaires and behaviour tests are commonly used in research and by kennel clubs for classifying breed characteristics (Goodloe & Borchelt, 1998; Wiener & Haskell, 2016; Wilsson & Sundgren, 1997). Several of them have been validated using statistical analysis and comparing results with behavioural tests. A prominent example is the C-BARQ questionnaire which is used in many studies (Hsu & Serpell, 2003; Serpell & Hsu, 2001).

The questionnaire is a fast and relatively cost-effective method of gathering large amounts of phenotype data from privately owned dogs. The quality of data will partly depend on owner expertise. It is however less optimal for finding individual variations than behaviour tests, due to a large number of assessors, which may reduce reliability compared to behaviour test with fewer evaluators. Owners have a subjective opinion of their dogs' behaviours which may introduce bias in the evaluation. There are also limitations to interbreed-comparisons since owners choosing specific breeds may have different expectations. What an experienced owner of one breed of dog feels is anxiety or pathologic, is likely not the same an experienced owner of an entirely different breed. An example of bias influenced by owner expectations.is airway disease in brachycephalic dogs. It has been shown that brachycephalic breeds have a high prevalence of clinical signs of airway disease. However, the proportion of owners of brachycephalic dogs who feel their dogs have "breathing problems" is significantly lower than the prevalence of reported clinical signs (Packer et al., 2012). This issue is likely not limited to breathing problems, but to numerous other potential pathologies, whether medical or ethological. It would be expected that growling would be evaluated differently in a guard dog (less serious) compared to in a family dog like a flat coated retriever. There are reasons to believe that the system for behaviour classification will influence the possibility to identify associated loci. Other questionnaire biases have been shown in different studies.

A study on human impulsivity found no correlation between genotype and questionnaire-based phenotypes but found significant results using test-based phenotypes. Suggesting some self-reporting bias (Eisenberg et al., 2007). Several factors may influence dog-owner scoring on a survey. Breed expectations, owner bias and recent untypical behaviour episodes. Cultural differences might also influence surveys. In a cross-cultural comparison of dog behaviour, Wan et al found that American owners rated their dogs higher on confidence and aggressiveness than Hungarian owners, an alternative reason for these findings may be a true difference in temperament between American and Hungarian dogs (Wan et al., 2009). Owner subjectivity bias in the questionnaire can possibly be diminished using specific phrasing and design of questions. Overall et al designed a noise-sensitivity questionnaire where owners were asked to pick from a list, a certain response the dog has to specific stimuli, but further research is needed to know the impact these methods have on inter-assessor repeatability (Overall et al., 2006).

3. **Observational studies**, where researchers observe the animals continually over a period of time, e.g. 24 hours, measuring how they interact with their environment. These studies are less common with companion animals and more common in kennels or research facilities. They are also more common in animal husbandry or wildlife. (Döring et al., 2016)

4. **Expert rating** where an observer with experience and competence on behaviour phenotypes rates the behaviour of individuals or breeds (Hart & Miller, 1985).

Genes associated with behaviour phenotypes.

Since it became apparent that all behavioural traits are influenced by genetics, there have been considerable scientific research into which genes affect different behaviour phenotypes the most. In the field of psychiatric genetics, candidate gene studies have been popular, and in 2004 the rate of published studies were about 1 per day (Munafo & Flint, 2004).

In a prospective longitudinal study, investigating why stressful experiences led to depression in some people but not others, a functional polymorphism was found in the promotor region of the serotonin transporter gene which significantly moderated the effect of stressful life events on depression. Where people with 1 or 2 copies of the polymorphisms were more prone to depressive symptoms after stressful life events.(Caspi et al., 2003)

Dopamine Receptor D4 (DRD4) polymorphisms have been linked to numerous psychiatric and behavioural phenotypes (Ptáček et al., 2011) e.g. attention deficit hyperactivity disorder (ADHD), substance dependency, stress-reactions and several other specific personality traits.

Difficulty identifying single loci associated with behavioural traits (missing heritability)

However, the results from meta-analyses on candidate gene studies have been largely inconclusive. (Munafò, 2006) with lack of repeatability being one major issue. (Rietveld et al., 2014) Replication has been the exception rather than the rule in most reviews of candidate gene studies. It is commonly believed that the reason for the inconsistent replications are low powered studies (small number of participants), and that candidate gene studies have problems with controlling for multiple hypotheses and controlling for “population stratification” – genotypes may covary with unobserved environmental factors such as ethnic cultures or religions.

Advances in technology in the last decade led to Genome Wide Association Studies (GWAS) which have been used to identify a very high number of loci associated with different inherited traits. GWAS is also more able to control for multiple hypotheses and also mitigate population stratification.

Using this technology; investigating variations in the human genome associated with behaviour yielded few significant results. Despite the high heritability reported for many behaviour traits, GWAS have consistently been unable to identify gene variations for personality (Bae et al., 2013; de Moor et al., 2012; Terracciano et al., 2010). The heritability of schizophrenia is high, but only part of it can be explained by known associated alleles, suggesting the existence of “missing heritability”. A genetic risk profile score based on 108 genetic loci reported by a large genome-wide association (GWA) study explained 7% of the variation on the liability scale (Stephan Ripke, 2014). Individual GWA studies have not suggested a gene of major effect (Riley & Kendler, 2006) But a meta-analysis of several GWAS’ identified association on chromosome region 2q and ten other regions, but similar to non-pathologic behaviour, there is evidence that schizophrenia has a complex inheritance and that hundreds of different genes each have a small but significant contribution to the risk of developing the phenotype (Purcell et al., 2009).

Compared to the high degree of heritability found in twin studies, this missing heritability has been a paradox in the field of behaviour genetics. Is the missing heritability due to bias in twin studies? Or, as it has largely been explained, is it due to the low frequency and small individual effect of each variation, thus needing a very large sample size to detect them.

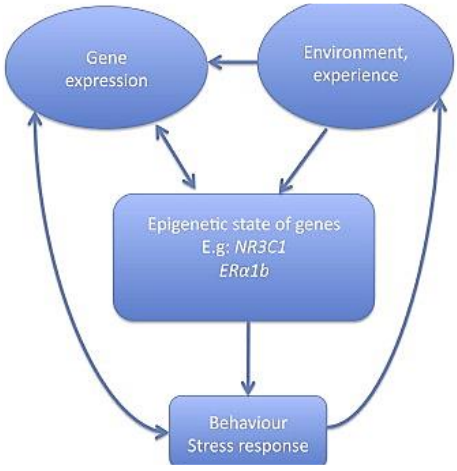
Genomic-Relatedness-Matrix Residual Maximum Likelihood (GREML), is a technique allowing estimation of heritability based on gene variants without needing to specify which specific variants are responsible for the heritability (Lee et al., 2011). Using GREML, Vinkhuyzen et al found ~45%

heritability of neuroticism and extraversion in humans (Vinkhuyzen et al., 2012). Also in "the big five" of personality: openness to experience, conscientiousness, extraversion, agreeableness and neuroticism (Power & Pluess, 2015b) these results have varied between heritability of 6-21%, in contrast to the high degrees shown in twin studies. Although twin studies are expected to show high degrees of heritability due to shared environments, the gap between the heritability estimates of different scientific approaches has been somewhat unexplained since the start of GWAS. However, using GREML and other sophisticated matrix-models we are beginning to close the gap between the heritability estimates of twin-studies and GWAS.

Epigenetic influence on behaviour.

Epigenetics describes heritable changes in gene function that do not involve changes in the DNA sequence itself. Changes like DNA methylation, histone modification and silencing of genes by non-coding RNA are the most important mechanisms. In an individual, certain environmental stimuli e.g. toxins, stress and exercise, have been linked to the binding of inhibitory or activating molecules, like methyl-groups, to the chromatin, thus reducing or increasing the expression of certain genes. This methylation of the chromatin illustrates how the environment can influence the expression of genes, which in turn influences the phenotype (Figure 3) (Allis & Jenuwein, 2016). Epigenetic silencing, e.g. DNA-methylation, has been shown to be inheritable (Holliday & Ho, 2002) which means that new epigenetic markers (like methylation) are transferrable from one generation to the next one. There is evidence that epigenetic mechanisms affect behaviour. One of the earlier studies on epigenetic influence on behaviour showed that the amount and type of nurturing provided by rat mothers for their offspring had an impact on how the rats responded to stress later in life. This was shown to be linked to the methylation of the promoter region of the glucocorticoid receptor gene NR3C1 (Miller, 2010). There is also evidence that epigenetics has an influence on human risk-taking behaviour (Kaminsky et al., 2008), drug addiction (Bönsch et al., 2004), and stress-response (Masterpasqua, 2009). However, the relationship between personality and epigenetics is still unclear. Even less investigation has been conducted on the effects of epigenetic inheritance on behaviour, but the number of studies is increasing, although they are almost exclusively using mice (Jensen, 2013)

Figure 3 - Jensen,2013



The canine model for behaviour genetics

There are indications that it may be easier to identify specific loci associated with complex traits, including behavioural traits in dogs, due to the potential loss of variation through evolution as well as the unique pedigree structure where dogs are separated in many breeds. Despite all dogs being members of the same species, each breed represents a distinct, highly genetically homogenous population with very low genetic heterogeneity within a specific breed. In addition, there are distinct differences between breeds, due to the selection and use of breeds for different purposes like family dogs, hunting dogs and guarding dogs.

Linkage disequilibrium is reported as being up to 100x higher in dogs than in humans, which gives longer haplotypes and likely means needing fewer genetic markers in a GWA study (Sutter et al., 2004). On the other hand, it could make it harder to move from a genetic marker to identifying the causative gene.

Dodman et al used GWAS to investigate SNPSs associated with the complex behaviour disorder Canine Compulsive Disorder (CCD) and flank sucking in Doberman Pinschers and found a SNP in CDH2 that correlated significantly with CCD (Dodman et al., 2009). Tiira et al performed a candidate gene study with this same locus on the CCD intermediate phenotype of tail chasing in Bull Terriers but found no association with the locus reported by Dodman. This is not surprising since different forms of CCD may have a different genetic background, and since there may be a locus heterogeneity between breeds (No association was found in a GWAS between bullterriers and tail chasing endophenotype.) This study had a limited sample size and may have been underpowered (Tiira et al., 2011).

Zapata et al found from a Genome Wide association (GWA) study using questionnaire-based phenotype that The IGF1 and HMGA2 loci variants predisposing to owner related aggression and small body sizes, were distinctly different from two loci on chr18 and X which predisposed to aggression towards unfamiliar humans and dogs (Zapata et al., 2016). This distinction between owner-related aggression as a different type than unfamiliar related aggression mirrors the heritability findings of Duffy et al, Van den Berg et al and Liinamo et al, but Zapata also found evidence that small body sizes correlate with the gene variants predisposing to owner-related aggression (Duffy et al., 2008; Liinamo et al., 2007; van den Berg et al., 2006),Bellamy et al found,, association between anxiety in Havanais and a SNP in exon 2 of Dopamine receptor D2 gene (Bellamy et al., 2018).

Sarviaho et al performed a GWA Study on German Shepheds for two phenotypes; noise sensitivity and fear of humans in novel situations. They found trait-significant loci on chromosome 20 for noise sensitivity, and on chromosome 7 for fear of humans in novel situations. These regions overlap human neuropsychiatric loci with candidate genes that are involved in dopaminergic and glutaminergic transmission. (Sarviaho et al., 2019)

Puurunen et al investigated physiological differences between German shepherds with varying ADHD-like behaviors. They found that tryptophane and kynurenic acid metabolites were significantly associated with ADHD-like behaviour. The metabolites are similar to earlier findings in human and rodent ADHD models.(Puurunen et al., 2016)

Aims of study:

Main goal

Study the association of selected candidate genes and behaviour traits in some dog populations

Sub goals

1. Study the genetic variation of 3 neurotransmitter genes in 6 different populations of dog. 33 populations classified as case/controls regarding noise sensitivity phenotype: Smooth haired collies, Nova Scotia Duck Tolling Retrievers and Irish Soft Coated White Terriers. Bichon Havanais were classified as case/control regarding anxiety phenotype. 1 population of Malinois did not have phenotypic data. 1 population of Belgian Malinois were classified as case/controls in different phenotypes using data collected for this study,
2. Investigate potential associations between potential variants and behavioural phenotypes in the study populations.

Materials and Methods

Selection of candidate genes for the study

Three genes previously reported to be associated with behaviour were selected for investigation: Serotonin receptor 2A(HTR2A), Dopamine Receptor D2 (DRD2) and Dopamine transporter (DAT).

Based on the history of serotonin-pathways involvement in many psychiatric and behavioural personality disorders and being the target of many psychiatric drugs e.g. SSRIs MAOIs, it was of interest to study possible associations between canine behaviour and genes involved in the serotonin-pathways. HTR2A was specifically selected partly because there has been comparatively little research into this gene in dogs, but evidence of association with behaviour in other species (Zhang & Stackman, 2015).

DRD2 was specifically selected on the basis of previous work done by Bellamy (Bellamy, 2015) where polymorphisms were found to be associated with an anxious phenotype in Havanais.

DAT was specifically selected on the basis of previous work done by Lit et al (Lit et al., 2013) where polymorphisms were found to be associated with aggressive intermediate phenotypes in Belgian Malinois.

Selection of dogs and DNA sampling

Three population of dogs with behaviour records, including different aspects of anxiety and noise sensitivity.

Belgian Malinois

Blood was sampled from 81 Belgian Malinois dogs at 10 weeks of age, bred and trained at a mine-detection training facility in Sarajevo by the Global Training Centre for Mine Detection Dogs (GTC) for the Norwegian People's Aid (NPA). The dogs were candidates for Mine detection dog training and all dogs passed a thorough behaviour puppy test. Blood samples were collected on site in Sarajevo, using standard EDTA vials. Samples were subsequently shipped from the Sarajevo site to a laboratory in Norway for DNA isolation. In addition, 40 Malinois dogs from the Norwegian police force were included in the study, to increase population size when investigating inbreed allelic variation. These dogs were not phenotyped.

Breeds with an observation on Noise sensitivity

DNA was sampled from blood samples collected in conjunction with a previous genome-wide noise sensitivity association study (Storengen, 2015). Privately owned dogs from three breeds were (Collies n=107, Irish Soft Coated Wheaten Terrier (WT) n=46 and Nova Scotia Duck Tolling Retrievers (Toller) n=33).

The selection of DNA samples were cases and controls based on results from a previous web-based questionnaire study on these populations (Storengen & Lingaas, 2015)

Bichon Havanais

DNA was extracted from blood samples collected in conjunction with a previous candidate gene study and master's thesis on social anxiety in Bichon Havanais. (Bellamy, 2015). 65 DNA-samples were selected. The dogs were classified as cases (N=32) or controls (N=33) based on owner questionnaires, interviews and provocation testing performed by Bellamy. The Havanais population represented two distinct groups with a clear phenotypic difference. Dogs were recruited through social media and Havanais-breed club newsletters. All Havanais were invited to participate in the

study, and anxious dogs were encouraged to participate. Breeders and owners were also contacted directly.

DNA isolation and quality control

DNA was isolated from blood samples using the E.Z.N.A® Blood DNA Mini Kit Protocol. See Appendix B, DNA isolation for details. DNA was isolated from buccal swabs using a standard protocol.

To ensure the isolated DNA-samples were of adequate quality; all the samples were subject to analysis of purity and concentration, using a NanoDrop Spectrophotometer.

The instrument was wiped with a dry paper tissue and 2µL of dH_2O were applied to calibrate, and then each sample was analysed, using 2µL each time.

Samples considered of poor quality were re-isolated and re-analysed.

Behaviour phenotypes

Malinois - phenotypes

Behaviour phenotypes were recorded as part of an "in-for-training" phenotype testing of puppies, which is a part of the training program of Global Training Centre (GTC) of the Norwegian Peoples Aids (NPA) training centre in Sarajevo. The purpose of the test was to select the best puppies for training to increase the success rate and reduce the cost in training. Puppies were tested at 10 weeks of age in an unfamiliar room with the tester being unfamiliar to the puppy. The tests were all recorded in a non-intrusive manner and the videos provided the basis for scoring the different aspects of the test. Scoring was performed by experienced handlers on site, based on video material. Results were scanned and converted into a commercial spreadsheet for analysis.

The behaviour test recorded several specific behaviours (Appendix A) that are believed to be associated with curiosity, interest in novel objects, hunting traits and to score different types of anxiety/confidence traits which are of great interest for these working dogs that may need to work under tough field conditions. Based on individual records, dogs could be classified in different behavioural classes and as phenotype cases/controls using the test description as the basis for defining the phenotype-criteria. (Appendix E). The detailed descriptions of the tests and instructions on how to score different behaviours provided the main basis for phenotyping.

Recording and classification of noise sensitivity

A web survey was conducted in collaboration with breed clubs. The survey included questions on varied topics concerning health, wellbeing and behaviour. The owners were asked to answer 4questions concerning their dogs' reactions to loud noises including gunshots, fireworks, thunderstorms and heavy traffic.

- Does your dog show signs of being fearful during loud noises/gunshots?
- 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 1-5

1. No signs
2. Mild signs
3. Moderate signs
4. Strong signs
5. Very strong signs

A dog was classified as fearful (case phenotype) if it had a score of minimum 4 in at least 1 of 4 situations.

Social anxiety (Bichon Havanais)

A standardized evaluation of the dogs' behaviour was performed for each dog. The evaluator first presented herself to the owner, ignoring the dog. The evaluator then approached the dog directly by bending down, holding one hand forward and calling the dog. Last, the dogs' reaction to gentle restraint at an examination table prior to DNA-sampling was registered. The tail position was noted at the time of initial greeting.

The dogs were observed and classified for three criteria (contact seeking, tail position and reaction to gentle restraint that physically supported and stabilized the dog, table 4). Dogs that displayed fearful behaviour in all criteria were classified as cases and dogs that displayed no fearful and only affiliative behaviour in all criteria were classified as controls. The same person (KB) evaluated all the dogs that were included in the study (Bellamy, 2015).

Table 4: Behavior traits for anxiety phenotyping

Trait	**Anxious**	**Control**
First contact with observer	Pulling away	Actively contact seeking
Tail Position	Down	Up
Reaction to gentle restraint	Strong avoidance	No avoidance or positive reaction

Identification of genetic variation in candidate genes

From each of the candidate genes, we aimed at sequencing functional parts of the genes in several dogs to detect genetic variation/variants with a minimum allele frequency.

Each gene was initially studied in 24 individuals to detect variation.

Primers were designed for coding regions, promoter regions (500 bp upstream of the first exon) and non-coding regions with genetic markers reported in literature with the aim of investigating the presence of variation in the population, and potentially non-random associations with phenotype.

Primers were designed using primer3plus.

All primer solutions were diluted to 5 pM/µL

5-Hydroxy Tryptamine Receptor 2A gene (HTR2A)

CanFam3.1 NC_006604.3 Ch22:4453715-4510934

HTR2A is a gene with 3 exons in human, which was not properly annotated in dogs (Canfam 3.1) (Ensembl) when the study was started. Based on comparative genomics we identified the location of the exons in dogs and primers were designed for the promoter region and for all three exons. We also amplified several intronic sequences containing SNP's found in literature (Appendix C) and two microsatellites we found in introns (Table 5, Appendix C). Two polymorphic microsatellites closely linked to HTR2A was used for the study.

HTR2A-MS1F	GGATCAGCTCTCCAACCAGT
HTR2A-MS1_R	TTACTGCTGGTTGCACCTTG
HTR2AmsCA17F	TGCACCGCAATGTTTATAGC
HTR2AmsCA17R	TTCAATCCGTGTTGTTGCAT

Table 5, excerpt

Dopamine Transporter gene (DAT)

CanFam 3.1 NM_001136500 Ch34: 11,210,939-11,246,784

Lit et al (Lit et al., 2013) investigated a Poly A marker and a Variable nucleotide tandem repeat (VNTR). We amplified and studied both polymorphisms using the same primers as in the original paper

Two microsatellites were identified by a thorough study of the DNA sequence. Both contain 2bp repeats; a GT16 microsatellite at IVS9 +2146 bp and a GT18 microsatellite 32.114 bp downstream of the last exon (exon16). (Table 6)

Using these 4 markers, we utilized the software *PHASE* for haplotype construction, and recombination rate estimation from population data (M stephens, 2005), to create an overview of the DAT haplotypes in the Sarajevo population.

Table 6: Primers used for investigating variation in DAT gene

Name	Sequence	Location	Ref
DAT Poly A (Lit) F	CAGATCAGACATTACTCTAACTATTGC	34:11,243,915	(Lit et al., 2013)
DAT Poly A (Lit) R	CCTTTTTCCCTGCTTGATG		
DAT GT16F	TGCCCTGTGATGAGTG	34:11,234,331	New, this study
DAT GT16R	GAGTTCCCCTTCCTGGAGTC		
DAT VNTR F	CTCCTGTGTCCCCGCTGTCTT	34:11,235,395-11,235,543	Lit et al
DAT VNTR R	GACAGAGCAGGGCAGGGAGG		
DAT GT18 F	ACTCGCACAGTCCACACTTG	34:11,278,899	New, this study
DAT GT18R	CATGGAACCTACCGCTGACT		

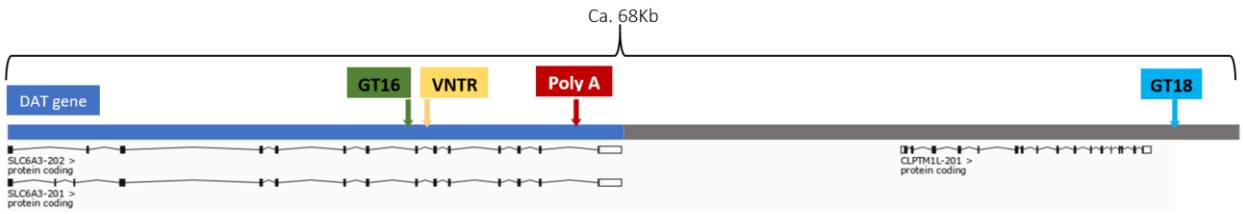


Figure 4 – Illustration of marker locations in Dopamine Transporter Gene

Dopamine Receptor D2 gene (DRD2)

CanFam3.1:CM000005.3 Chromosome 5: 19,732,880-19,795,252

Primers for sequencing exon2 were copied from Bellamy with the aim to investigate any variation in the Sarajevo population(Bellamy, 2015). (Table 7).

DRD2 exon 2 F	ACTCGCACAGTCCACACTTG
DRD2 exon 2 R	CATGGAACCTACCGCTGACT

Table 7

Sequencing and fragment analysis

All markers were tested 10 different DNA samples together with primers on a standardized PCR-protocol (Appendix D). PCR-products were subsequently tested using gel-electrophoresis to test whether the PCR had sufficient yield. (Appendix D)

All PCR-assays samples were run on a standardized PCR protocol (Appendix D)

Fragment-lengths were analysed using Genemapper 5.1. (AB). Products with peaks lower than 200Hz were disregarded as they could not be reliably differentiated from background noise.

Alleles were named after their PCR-fragment lengths and corresponding loci e.g. “HTR2A MS1 290” allele is the 290 base pair long fragment of the MS1 locus (ch22:4.459.820) of the HTR2A gene.

SNPs were visualised / analysed using Sequencher software (GeneCodes), using CanFam 3.1 as reference sequences.

Statistical analysis

Associations were estimated as odds ratios using MedCalc for Windows, version 18.5 (MedCalc Software, Ostend, Belgium).

$$OR = \frac{a * d}{b * c}$$

With Standard error being

$$SE\{\ln(OR)\} = \sqrt{\frac{1}{a} + \frac{1}{b} + \frac{1}{c} + \frac{1}{d}}$$

And 95% confidence interval

$$95\%CI = \exp(\ln(OR) - 1.96 * SE\{\ln(OR)\})\ to\ \exp(\ln(OR) + 1.96 * SE\{\ln(OR)\})$$

Standard normal deviate (Z) being

$$\frac{\ln(OR)}{SE\{\ln(OR)\}}$$

And P-value being the area of normal distribution falling outside ± Z

Results

Phenotypes from description of puppy test

The different observations in the test were included in the following phenotypes (table 8, Appendix E). Shy/Bold phenotype, Playful phenotype, High search intensity phenotype, Fearful of humans phenotype, Startle reflex, Interact chain and Cautious/curious phenotype.

Subgoal 1: Identification of genetic variation

Genetic variation in 3 neurotransmitter genes in GTC Belgian Malinois

DRD2

The following SNPs were identified in or close to exon 2 (table 9) Wild type marked with *

Allele	Location		Status
G*/A	5:19782497	Intron 1	Annotated
C*/T	5:19782666	Exon 2	Annotated synonymous variant
T*/A	5:19782773	Exon 2	Not annotated
T*/C	5:19782828	Exon 2	Annotated synonymous variant
T*/C	5:19782940	Intron 2	Annotated

Table 9

HTR2A

For HTR2A we were not able to detect variation in the sequenced exonic parts of the gene, indicating that there was low variation in the coding parts of this gene. For unknown reasons, we had however also difficulties in amplification of parts of the gene which may have been due to incorrect sequences in the database. Since we principally were interested in associations, and not functional effects, we identified two microsatellites (MS1 and CA17) in the gene, which both showed variation within the population.

MS1 had 4 alleles, lengths ranging from 284 to 290, with 288 being the most common. (Table 10)

MS1 Allele	Allele frequency
284	6
286	2
288	88
290	62

Table 10

DAT

We found variation in all 4 different markers on the DAT gene. (Table 11) From the data we identified 16 different haplotypes in our population. (Table 12)

DAT GT16		DAT Poly A/AAA		DAT GT18		DAT VNTR	
Allele variant	Frequency	Allele variant	Frequency	Allele variant	Frequency	Allele variant	Frequency
385	63	232	86	322	82	268	69
391	38	250	31	342	78	308	84
393	32	262	35				
395	11						
397	6						

Table 11

DAT Haplotype name	DAT Haplotype				Frequency
	GT16	**AAA**	**GT18**	**VNTR**	
1	385	268	232	342	1

2	385	268	262	322	18
3	385	308	232	322	1
4	385	308	232	342	46
5	385	308	250	342	2
6	391	268	232	322	32
7	391	268	262	322	7
8	391	308	250	342	1
9	393	268	232	322	1
10	393	268	262	322	10
11	393	308	232	342	1
12	393	308	250	342	24
13	395	268	262	322	2
14	395	308	232	322	10
15	397	268	262	322	1
16	397	308	250	342	5

Table 12

Genetic variation of 2 neurotransmitter genes in Norwegian Police Force Belgian Malinois dogs

24 dogs were genotyped on the DAT poly-A, and DAT VNTR loci, (Table 13) and 40 dogs were genotyped on the HTR2A MS1 locus.

DAT

In the 23 dogs successfully genotyped, 4 different haplotypes were identified with haplotype 2 being the most prevalent. (Table 14)

AAA			**VNTR**	
Allele	Frequency		Allele	Frequency
232	28		268	19
262	14		308	27
250	4			

Table 13

Haplotype	**AAA**	**VNTR**	**Frequency**
1	232	268	5
2	232	308	23
3	250	308	4
4	262	268	14

Table 14

HTR2A

In the 40 dogs genotyped, 2 alleles were identified for MS1. A 288bp long fragment, and a 290bp long fragment. (Table 15 and 20)

HTR2A MS1	
Allele	Frequency
288	31

290	49

Table 15

Genotype	frequency
288/288	7
288/290	17
290/290	16

Table 20

Genetic variation in candidate genes in the study populations

Nova Scotia Duck Tolling Retriever

HTR2A and DAT loci showed variation in the population. (Table 21) DAT VNTR primers did not yield PCR-product.

HTR2A MS1 allele	Frequency	DAT GT16 allele	frequency	DAT Poly-A Allele	Frequency	DAT GT18 allele	Frequency
284	9	*385*	7	*232*	45	*322*	39
288	30	*395*	12	*250*	21	*342*	25
290	27	*397*	20			*344*	2
		403	24				

Table 21

Irish Soft Coated Wheaten Terrier

HTR2A and DAT loci showed variation in the population however there was greater homogeneity in WT compared to NSDTR (Table 22) GT18 primers did not yield PCR-product

MS1 allele	Frequency	VNTR allele	Frequency	GT16 allele	Frequency	Poly-A allele	Frequency
284	1	*308*	80	*363*	10	*232*	78
288	73	*268*	10	*385*	2	*262*	10
290	16			*391*	4		
				395	60		
				391	4		

Table 22

Collies

Relative frequencies of alleles shown for each marker. (Table 23)

Table 23

MS1 allele	Frequency	VNTR allele	Frequency	GT18 allele	Frequency	Poly-A allele	Frequency
288	2	*308*	192	*340*	3	*232*	21
290	46			*342*	45	*250*	169

Table 23

GT16 and further investigations into Collie variants were discontinued due to limited allelic variation.

Bichon Havanais

The Bichon Havanais phenotypes were based on general anxiety phenotypes (bold/anxious). The bold phenotype was labelled as control, and anxious dogs were labelled cases.

A selection of the 16 most clearly bold, and 16 most clearly anxious Havanais dogs were tested for association to the DAT Poly-A, GT16 and GT18 loci. We also tested for in the HTR2A MS1 Locus.

In DAT we found variation in the GT16 and the GT18 loci, 5 different alleles were present in each. We found no variation in DAT poly-A polymorphism. (Table24)

HTR2A MS1 allele	frequency	GT16 allele	frequency	GT18 allele	frequency
284	11	385	22	322	21
288	8	391	8	328	11
290	32	395	8	324	1
292	7	397	10	342	21
		401	11	348	3

Table 24

Subgoal 2 - Association between variants and phenotypes

GTC Belgian Malinois – (puppy behaviour test)

1. Shyness/Boldness

We found increased numbers of haplotypes containing the 385 allele of the GT16 microsatellite in dogs classified as bold compared to haplotypes without the 385 allele (OR=2,11, n.s). (Table 25)

	Bold (Sum score ≥ 7)	Shy (sum score ≤5)
DAT Haplotypes 1-5 (GT16; 385)	38	12
DAT Haplotypes 6-16 (GT16; no 385)	36	24

1 Odds ratio	2.11
95 % CI:	0.91 to 4.86
z statistic	1.76
Significance level	P = 0.078

Table 25

With the shy group ≤6, and bold group (sum=8) we got following results. (Table 26)

	Bold Sum = 8	Shy Sum ≤6
DAT Haplotypes 1-5	37	30
DAT Haplotypes 6-16	35	58

1a Odds ratio	2.04
95 % CI:	1.07 to 3.87
z statistic	2.19
Significance level	P = 0.028

Table 26

No significant association was found between Shyness/boldness and DRD2 or HTR2A.

2. Playfulness

In comparing the playful phenotype to HTR2A we found an association between the MS1 microsatellite and playfulness. The 290 allele of the MS1 microsatellite had a twofold OR of being playful (Table 27)

	Playful	Non-playful
MS1 290 allele	31	19
MS1 non-290 alleles	24	33

2 Odds ratio	2.24
95 % CI:	1.03 to 4.87
z statistic	2.04

	Significance level	P = 0.04

Table 27

No significant correlation was found between playfulness and DAT or DRD2 haplotypes

3. Search intensity

Search intensity was nominally associated to HTR2A; where the 290 allele of the MS1-allele had increased odds (OR=2,67) of having a high search intensity compared to other alleles of the MS1 microsatellite. (Table 28)

	High search intensity	Low search intensity
MS1 290 allele	32	12
MS1 non-290 allele	24	24

3a Odds ratio	2.67
95 % CI:	1.11 to 6.37
z statistic	2.205
Significance level	P = 0.0275

Table 28 – Search intensity study design A

Case/control design B showed the results summarized in table 29

	High search intensity	Low search intensity
MS1 290 allele	32	22
MS1 non-290 allele	24	56

3b Odds ratio	3.39
95 % CI:	1.64 to 6.99
z statistic	3.311
Significance level	P = 0.0009

Table 29 search intensity study design B

No significant correlation was found between search intensity and DRD2 or DAT haplotypes.

4. Fear of humans

In comparing the "fearful-of-humans" phenotype to DAT correlation was found between haplotypes bearing the 385 allele of the GT16 microsatellite, and the odds of being fearful or not fearful of humans (Table 30)

	Non-fearful Score = 4	Fearful Score ≤ 3
DAT Haplotypes 1-5	38	30
DAT Haplotypes 6-16	36	58

4 Odds ratio	2.04
95 % CI:	1.08 to 3.84
z statistic	2.205
Significance level	P = 0.027

Table 30

When excluding individuals scoring >1 from the fearful group, thereby increasing the phenotypic gap between cases and controls, similar OR was shown (OR=1.96), but power was reduced (P=0.19)

5. Loud noise aversion

No significant correlation was found with DRD2 exon2 variations, DAT Haplotypes or MS1 alleles, in this dataset.

6. Interact chain

An association between interaction with chain was found to HTR2A: Dogs with the 290 allele had increased odds (OR=3,67) of having high chain interaction. (Table 31)

	High chain interaction	Low Chain interaction
MS1 290 allele	37	9
MS1 non-290 allele	37	33

Table 31

6 Odds ratio	3.67
95 % CI:	1.54 to 8.72
z statistic	2.93
Significance level	P = 0.003

7. Cautious/Curious

For the cautious/curious phenotype we found an association with HTR2A. Again the 290 allele was positively associated with curiosity (OR=3.05). Correlation was found between the 290 allele and the odds of being in the cautious or curious group (Table 32)

	Curious	Cautious
290 allele	30	21
not 290 allele	22	47

7 Odds ratio	3.05
95 % CI:	1.43 to 6.48
z statistic	2.903
Significance level	P = 0.0037

Table 32

Breeds with an observation on Noise sensitivity

Nova Scotia Duck Tolling Retriever

No significant association was found between HTR2A (MS1) or DAT (Poly A) allele variants and phenotype. For the DAT GT16 microsatellite locus the 403bp allele was associated with Case (Noise sensitivity) phenotype. (Table 33) GT18 locus and VNTR locus was not investigated.

DAT GT16	case	control
403	17	7
not 403	15	24

Odds ratio	3.88
95 % CI:	1.30 to 11.57
z statistic	2.438
Significance level	P = 0.0148

Table 33

Irish Soft coated Wheaten Terrier

An association was seen between the MS1 microsatellite locus (HTR2A) and noise sensitivity-phenotype. The 290 allele was associated with protective effect (Table 34)

Htr2a MS1	Controls	Cases
290	13	4
Not 290	36	36

Odds ratio	3.25
95 % CI:	0.96 to 10.92
z statistic	1.90
Significance level	P = 0.056

Table 34

DAT allele variations showed no significant association with phenotypes. One allele was distributed in a seemingly non-random pattern; however, odds ratio was not significant at 5% (Table 35)

DAT poly A	control	Case
262	7	3
Not 262	41	37

Odds ratio	2.1
95 % CI:	0.51 to 8.74
z statistic	1.02
Significance level	P = 0.30

Table 35

Boldness / general anxiety in Bichon Havanais

For the HTR2A MS1 locus, we found a non-random, but non-significant distribution of alleles in cases and controls, with a high odds ratio comparing cases and controls at borderline significance. Because of this, we included an additional 16 control individuals and 20 case individuals, all phenotyped with the same methods. Results showed an odds ratio over 3, but not significant at p=0.05. (Table 36). DAT alleles were distributed randomly among cases and controls.

HTR2A MS1	Anxious(Case)	Bold(Control)
292	10	3
Not 292	58	55

Odds ratio	3.16
95 % CI:	0.82 to 12.09
z statistic	1.68
Significance level	P = 0.09

Table 36

Discussion

In the present investigation, we aimed to study associations between specific candidate genes, reported to be associated with behaviours in other dog breeds and species, and recorded shyness/boldness/anxiety-behaviours in dogs. We collected samples from anxiety-related traits from one breeding unit of working dogs and samples from four breeds of family dogs.

In the breeding unit, a systematic puppy-testing protocol secured thorough observation of several behaviours, and a number of these could be interpreted as having a relationship to anxiety. The underlying thought was; if a dog that is afraid of a new environment or person in the test room, will it lose the ability to perform/show natural explorative behaviours? One of the traits studied in the breeding unit was shyness/boldness which was defined as the sum of the first two tests: “Investigate Room”, and “Investigate tester”.

Shyness/Boldness

The first two tests in the series were administered to all puppies. These two tests quantify aspects of how the pups react to and interact with new environments and humans. These two tests are likely the closest approximation to any inherent boldness/shyness in the dogs since they are the first tests administered, before any potential confounding influence of other previous tests. There is little human or environmental interaction , as the pup is simply placed in a new environment and its reaction is quantified.

When comparing bold pups (scoring combined 7 or 8 to shy (scoring 5 or lower), we found an association with DAT-alleles/haplotypes (OR=2.11). Excluding pups scoring mid-levels (sum=6), has the effect of separating the phenotypes more clearly from each other, at the cost of reducing the number of included dogs and the statistical power. The estimated OR was not statistically significant at 0.05 (p=0.078).(

The results were borderline and sensitive to small changes in the classification used; When we used the top scoring pups (Sum=8) compared to pups scoring 6 or lower, thereby keeping similar degrees of phenotype separation by excluding mid-level pups (sum=7), , results were statistically significant (p=0.028). The changes in threshold for case and control, illustrate the importance of correctly

classifying behaviour-phenotypes and how results are sensitive for small changes in classification and inclusion criteria. The results also illustrate how the significance of the odds ratio calculations are heavily dependent on size of included material. The results also imply that DAT may be associated to the boldness/shyness traits in the Belgian Malinois. The change in significance levels when increasing bold-phenotype threshold illustrates that behaviour-gene associations are sensitive to how the behaviours are scored, and the interpretation (and inclusion criteria) of cases and controls. There are however several studies in different species supporting a potential role of dopamine pathway genes in various forms of anxiety (Ptáček et al., 2011; Riva et al., 2008). In dogs, Riva et al demonstrated higher plasma levels of dopamine in anxious dogs, compared to non-anxious. DAT polymorphisms specifically, have been associated with PTSD in humans (Segman et al., 2002).

These results support the hypothesis that the variants of the dopamine gene may influence on a dogs' reaction to novel situations. Bellamy found similar results, namely DRD2 gene polymorphism being associated with anxiety in Bichon Havanais. Shyness/Boldness may also be related to novel-seeking behaviour, which has been associated with polymorphisms in DRD4 in humans (Pogue-Geile et al., 1998).

It is however, important to remember that the studied microsatellites are not expected to have a functional effect on the phenotype. The potential functional polymorphisms could be in the DAT-gene, in regulating sequences, and functional alleles in other closely linked genes could also be involved, due to the long LD in dogs.

Playfulness and Search Intensity

Playfulness was defined as the average score on "active ball", "passive rug", "active rug" and "active bag". These three toys (ball, rug and bag) were all introduced in the same manner. First passively on the ground, then actively move it around and lastly quickly hide it to score search-intensity. Of the three toys, only the rug was familiar to the pup, which is why passive ball and passive bag was eliminated from playfulness phenotype, as these would likely be influenced to a greater degree by the pup's caution/curiosity to foreign objects, which may mask any playfulness the pup has with familiar objects. In other words, a pup may be playful with familiar objects, but have a varying caution to unfamiliar situations, which is why passive ball and passive bag was not included in the phenotype. After familiarizing the pups with the object, the active test would be a closer approximation of playfulness, although still be somewhat influenced by the dog's caution and curiosity.

Playfulness showed an association with HTR2A MS1 microsatellite. Dogs with the 290 allele had significantly ($p=0.04$) higher odds ($OR=2.24$) of being phenotyped as playful, rather than non-playful.

Search intensity was measured as the length of time, and the displayed intensity with which, the pup searched for the disappeared ball/rug/bag. High search intensity phenotype were dogs with an average of 4 on the search intensity traits, i.e. only the highest score possible in all 3 tests. Low search intensity was defined in two ways. Design A was defined as dogs scoring 3.33 or lower, thereby excluding all the dogs in between (n=20). This was done with the aim of separating the phenotypes to increase the difference between cases and controls. However, this sacrifices power in the study, so another design - design B - included all non-top scoring dogs in the Low search intensity phenotype, thereby increasing the number of individuals and power.

Search intensity results also showed a significant association between high search intensity and the 290 allele when using either study design A or B. Study design B compares the most intensive searchers with what is likely the average dog. Minimizing the distance between case and control

phenotypes, will possibly reduce the odds ratio but with the possible benefit of increasing power. In this case, both power and OR increased when comparing design A (OR=2.67, p=0.0275) to design B (OR=3.39, p=0.0009). This might be due to the 290 allele being in LD with a variant with strong effect on phenotype. Serotonin pathways have been implicated in motivation and playful behaviour in other animals, which is concordant with these findings (Siviy et al., 2011).

Search intensity and playfulness had a high correlation (R^2=0,84) in the puppy test, which implies the two traits have shared motivation. (Figure 5)

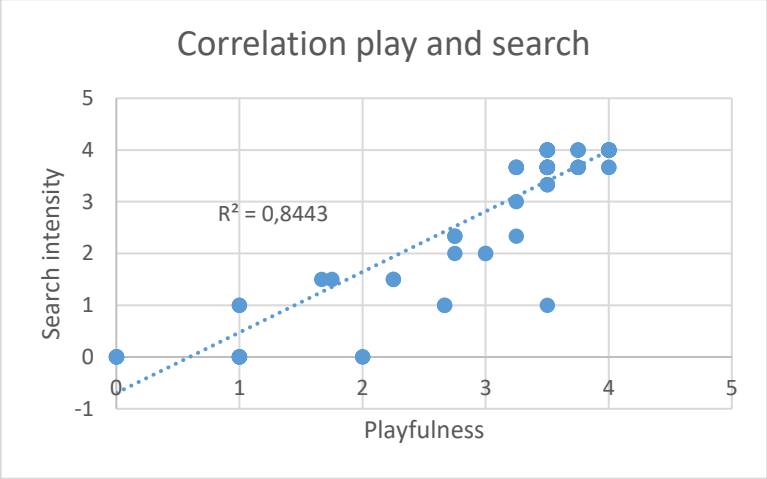


Figure 5

One possible hypothesis is that the interest in the toy is what motivates the search after it's disappearance, i.e. if the dog is not motivated to interact, it's not motivated for search. Another possible and supplementary hypothesis is; both traits are motivated by an underlying hunting or predatory desire/instinct.

In this investigation, playful behaviour was significantly associated with a microsatellite closely linked to a serotonin locus.

Playful behaviour has been defined and investigated differently in behavioural-studies (Rooney et al., 2000), and the motivations for play are not fully known. It's commonly agreed that one likely benefit is practice for behaviours that are useful later in life, e.g. hunting skills. Serotonin pathways are widely distributed in the body in all bilateral animals, and in many plants and fungi. Serotonin is also involved in a wide variety of behaviours (Frazer & Hensler, 1999). Based on this, one can predict some modulation on play behaviour from serotonin. Investigations into mammalian playfulness and serotonin have shown evidence of association. Several studies in rats showed a decreased play-behaviour at increasing serotonin tone (B. & J., 1997; Homberg et al., 2007) and one study investigating rat play-behaviour

under the effect of a specific HTR1A agonist, corroborated these findings, showing that play-behaviour decreased with high-doses of the agonist (Siviy et al., 2011).

Fear of humans

"Fear of humans" is one of the traits recorded with a clear association to anxiety traits. The phenotype was defined by the score on test element 2: Interaction with the tester. The aim was to

investigate if there was a difference between general anxiety-related behaviour and a specific aversion of humans/strange people. Studies have shown a difference between the aggression types and shown distinct subcategories of one type of aggression directed towards strangers, and a different type directed towards owners, and that their genetic influence is likely separate (Duffy et al., 2008; Zapata et al., 2016).

We identified a significant association between a DAT linked microsatellite (GT16), included in haplotypes 1-5, and the "fear-of-humans" phenotypes. These findings support other investigations by Bellamy and others, who found that Dopamine pathways are associated with anxiety or aggressive behaviour in Malinois, other breeds and other species (Bellamy, 2015; Lit et al., 2013) (Colombo et al., 2013).

This association could be influenced by the investigate room trait due to the high correlation between Test element 1 (investigate room) and test element 2 (investigate tester) ($R^2 > 0.8$) (Figure 6). It is possible stranger aversion and novel environment aversion are two separate phenotypes with separate genetic influence, but these results show that either the traits likely show some common genetic influence, or the traits influence each other in such a degree as to mask any possible separateness. For instance, the dogs may be so anxious from being placed in a new environment that any possible interest in the tester is masked by the general anxiety. The causal relationship is unknown in this instance. A degree of interest or comfort around the strange tester, may also put a general novel-environment anxious dog at ease and make it more likely to explore the room. In summary, the "fear of humans" phenotype is associated with a DAT polymorphism, but its close correlation with the previous test (investigate room) needs to be considered when interpreting the results due to likely influence from the previous test on the subsequent test.

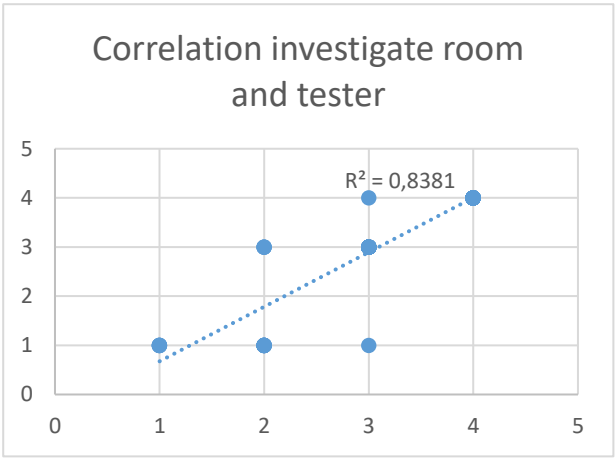


Figure 6

Startle reflex

The metal chain test characterizes the dogs' immediate reaction to a special loud, unknown noise or startling stimuli. This test is probably not directly comparable to noise tests in other behavioural tests, where a gunshot is more commonly used. The chain-noise is quite loud, and when combined with the sensation of chain-impact on the ground, and how close the origin of the sound is, it was likely a useful tool for triggering a startle response, moderately overlapping with a general noise aversion.

Results showed no significantly uneven distribution of phenotypes and genotypes of any of the tested loci. However, 19 dogs were not exposed to this test. This is because if a dog scored poorly early in the protocol, it would "fail", and the test would be aborted without completing the rest of the subtests.

Interact chain

The interact chain trait observes the dog's reaction to the object causing the startling stimulus. Our results showed an association between interaction with the chain and HTR2A. The test is likely both influenced by the dog's proneness towards startled behaviour, the degree of startle reaction, but also a general interest in new objects. The general interest in the new object is likely a large influence on which dogs interact with the chain, this also illustrates a bias in the total behavioural test. The 290

allele is already shown to be associated with interest in new toys. This is likely contributing to why 290-dogs are more prone to interacting with the chain. This implies the test is not actually scoring the dogs' reaction to startling, but rather it's playfulness or neophilia. Which suggests the test might need to be revised, seeing as it might not be testing what it is attempting to test (startle reaction).

The degree of interaction when compared to general interaction with the 3 other toys i.e. the playfulness phenotype, is lowered in the chain-test. Meaning, dogs who averaged 3's and 4's in the playfulness tests, score generally lower, i.e. 2's and 3's when interacting with the chain.

A likely cause is the startle-effect from the chain, just moments before the dog is evaluated on the interaction. This illustrates a bias in this test and behavioural tests in general; earlier test moments affect subsequent observations. Another possible explanation for the discrepancy is that the dog is not interested in the chain as a toy. This is less likely considering the similar direction of the "Playful" trend line, and the "Interact chain" trend line, meaning if the dog didn't consider the chain a toy, there would be no correlation with playfulness trend line and interaction trend line. (Figure 7)

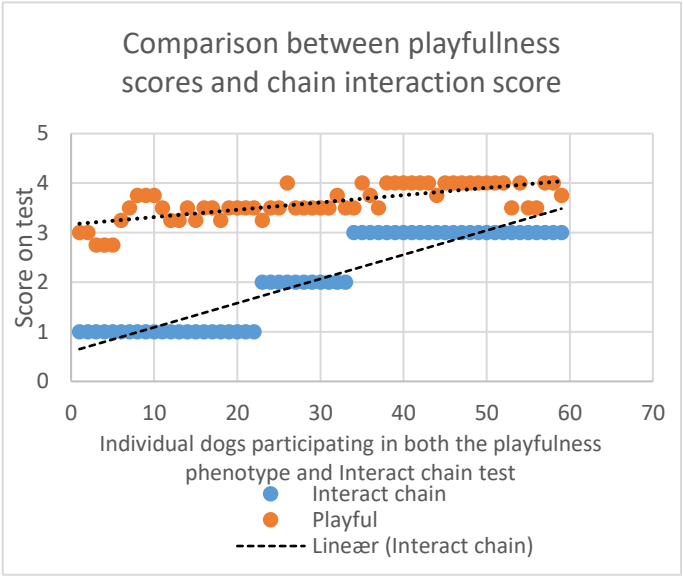


Figure 7

These findings imply the playful phenotype is quite a strong phenotype. Meaning it is only slightly influenced by other stimuli, and confounding influence affects all individuals equally, as evidenced by the correlating chain interaction and playfulness regression lines in figure 7

Cautious/Curious

Cautious/Curious phenotype was defined as the reaction to a passive ball. This ball was unfamiliar to the dog. Observing the dog's behaviour would therefore, yield a characterization of the dog's curiosity towards an unfamiliar but non-threatening object, in a novel environment. A passive bag was not included as it was considered to be a stronger phenotype early on in the test, as the dog has not been introduced to several new stimuli, and therefore the first passive unfamiliar toy test would be a better approximation of the curiosity. When the bag was introduced, the dog would already have been exposed to other toys, which it could have learned was not threatening, and therefore the bag-test was a less strong approximation of curiosity, than the ball.

Results showed an association of the trait with the HTR2A. The higher interest in balls in dogs carrying the 290 allele, implies that interest in the *passive ball* is motivated by the same mechanisms that motivate the interest in the *active ball*, and the *search after disappearance*. Any confounding influence from the "investigate room" + "investigate tester" subtests on these results, is likely minor, as they did not show any association with the HTR2A allele, but rather with DAT haplotypes. (See shyness/boldness)

Preference for new toys (neophilia) is not only anecdotally common, but there is also evidence for neophilia in adult dogs (Kaulfuß & Mills, 2008).

However, it's possible that neophilia is learned behaviour, and there is little evidence for inherent neophilia in puppies. The GTC behaviour test does not investigate the puppies' preference for a new

toy over a familiar one, but it does investigate how it reacts to a non-threatening introduction of a new object.

Several of the tests can be viewed as toy/object related (playfulness tests + chain interaction), and the results from all these tests are associated with the HTR2A MS1 290 allele. I.e. dogs with the 290 allele were more prone to interacting with the ball, the rug and the plastic bag. They were also more interested in searching for the toys after their disappearance, and they had a higher degree of interaction with the chain, however, the general shyness/boldness of the dogs was not associated with the 290 allele or any of the HTR2A MS1 alleles. This strong association between toy interest and certain HTR2A alleles, provide the foundation for a hypothesis that interest in toys is not only heritable, but also influenced by serotonin pathways, in this population of Belgian Malinois, and possibly other breeds as well. There is corroborating evidence for this possible association in rats (B. & J., 1997; Homberg et al., 2007). Little is known or published on this association on other breeds or species, and further investigation is needed to increase knowledge about this association.

In the current study, we have studied the association of three genes with different test/expression of anxiety and other traits. When testing for several random variables it is important and usual to consider correcting for multiple testing and applying e.g. Bonferroni correction. This means that the significance threshold is changed by dividing the set point of significance (e.g. 0.05) with the number of hypotheses tested (McDonald, 2009). Bonferroni testing is also meant to avoid detection of false associations, among multiple random tests. A Bonferroni correction assumes the independence of the individual hypotheses. In our case, groups of tested markers are closely linked and in LD and the correction of significance for number of SNPs would be too conservative. Applying a Bonferroni correction will increase the chance of making a type II error, i.e. falsely retaining a null hypothesis (that there is no association with gene variants and behaviour phenotypes).

The correction for multiple-hypotheses is still important, and in this study some of the "border-line" significance might disappear after Bonferroni correction. A correction may also create a statistical bias. An alternative approach is described by Perneger: "*Describe the method and approach, and the reasoning behind it, which should enable the reader to reach a reasonable conclusion without the help of Bonferroni adjustments*" (Perneger, 1998). In this study, it is important to remember that even if several tests are performed, they are not essentially random. Tests selected are based on prior hypotheses and an a priori likelihood of association. In other words, the selected genes are reported to be associated with anxiety/behaviour traits and most of the discussed and studied traits have a reported component of anxiety.

The studies of noise sensitivity in three breeds of family-owned dogs showed interesting associations. Nova Scotia duck tolling retriever showed significant ($p = 0.0148$) association to DAT (GT16). When viewed in light of the GTC results (shyness/boldness association) this is not surprising, seeing as noise sensitivity is linked with a generalized anxious behaviour phenotype, and dopamine-allelic-variations was linked - in the GTC population - with phenotypes related to anxious behaviour.

In this material, several hypotheses were tested. Case/control phenotypes were compared with multiple but closely linked genetic markers, which should be kept in mind when interpreting results. In light of this, using a simple Bonferroni correction, the significance levels would be $0.05/4 = 0.0125$, which means p-level was close to significant with a likely over-conservative Bonferroni correction.

Irish soft coated wheaten terrier

No significant associations were found between noise sensitivity and any of the polymorphisms investigated. Only HTR2A showed tentative association with close to significance ($p=0.056$) The study

power on this breed was low, due to the limited number of available individuals, but is also possible it's a spurious correlation, especially if multiple testing is concerned.

However, the fact that the allele of HTR2A (290 bp), is also associated with several traits in the GTC dogs (playfulness/search/curiosity/chain interaction …), that seems associated with the non-anxious type phenotype, is of interest. In the GTC dogs, the 290 allele was associated with non-anxious behaviour (playfulness). This could indicate that the 290 allele might sit on an evolutionary conserved haplotype/be in strong LD, with variants that affect the phenotype towards a non-anxious phenotype. However, this material is too small to draw any conclusions. The fact that the WT breed show association with a different allele than the Toller breed could also indicate a spurious association or different marker-trait-haplotypes in different breeds. And to reiterate, the material showed only a borderline association, also without correcting for multiple testing.

Due to the lack of variation of DAT and HTR2A markers in the collie population, no association-study was possible for those markers. DRD2 was not investigated,

We were not able to identify a significant association between any of the investigated genes and the general anxiety and the recorded "social anxiety" in the Havanais. However, there was a skewed distribution of alleles of HTR2A also in this breed the 292 allele was most frequent in the anxious/case group, but the frequency of the potentially associated allele was very low. This may indicate that there is no association between these alleles in this population. However, the skewed distribution is interesting, and in case there should be any functional anxiety-associated alleles closely linked to HTR2A, they may sit on different haplotypes in different breeds/traits. In this context, it is important to note that the frequency of noise sensitivity, as defined by the owners, is very low in Havanais.

It is also important to note that the dogs are phenotyped for different behaviours that may not be directly comparable. Noise sensitivity and general anxiety are distinct disorders, even if they tend to be correlated. It is possible for instance, that the anxiety in Havanais has an entirely different genetic influence than noise sensitivity in other breeds. It is also possible that the genetic influence on noise sensitivity in other breeds is also influencing the boldness/shyness or playfulness in the GTC dogs.

Summary

Variants of SNPs and microsatellites in or closely linked to three candidate genes of behaviour, HTR2A, DAT and DRD2 were studied in three population of dogs with records of behaviour phenotypes

Most of the selected markers showed variation in the study populations and the markers are probably well suited for studies for similar studies in other populations

For most of the markers/phenotypes we were not able to show significant associations between markers and the behaviour phenotypes, but a few showed borderline significance

The study supports an association between HTR2A gene and play/search phenotypes in the mine detection dog population (OR=2,24, p=0,04) but the results has to be evaluated in the context of the number of markers studied/multiple testing and should be repeated in a bigger material.

The study also supports that boldness in these dogs might be associated with variations in the DAT gene; In the NSDTR noise sensitivity was associated with a genetic marker closely linked to DAT.

The study has confirmed that the use of dogs phenotyped for behaviour traits is well suited for the study of genetics of behaviour.

Further research and investigations are required to reveal potential associations and clarify the relationship between phenotype and specific candidate genes.

Limitations of the study

Some limitations have been discussed consecutively, however, it may be useful to summarize the major limitations of the study.

1. The behavioural test at GTC was aborted before completion if the test scores were low in the first tested traits. As a result, a large portion of the puppies didn't finish the last half of the test, this reduces the power when investigating phenotypes expressed in the latter half of the test. GTC places a high value on search intensity when it comes to determining eligibility for further training. Only the dogs with an average of 4 in the search intensity tests received an after-test remark (on the form) that the dog will be a good detection dog, and dogs scoring mid-range on this test had comments that were uncertain e.g. "possible donation", "follow up", "Could be ok". (Table 37) Dogs scoring low on this test failed the examination and were donated.
2. DNA-samples were initially just collected from dogs that passed the puppy test. Many potential cases were therefore not included in the material, which reduced phenotype contrast and number of samples in the material
3. Not all measurements had a linear relationship from lowest score to highest score. In some measurements, the 1s and 3s measurements of different behaviours than the 2s and 4s. For example, in test 2, where they measured interaction with the tester. A score of 1 was no contact or aversion from the tester, and a 2 was overly attached to the tester with no independence, and then 3s and 4s were independent but interested in the tester. Which show that in this test, and some others, there is not a linear relationship from 1 to 4 of "degree of the trait". They were included in the same measurement because they utilized the same tool e.g. a ball or rug to measure, and the 1s and 2s were less desirable even though 1s were puppies who were completely disinterested in the ball and a score of 2 would be overly interested in the ball. The test wasn't designed to rate different expression of phenotypes from weak to strong, but rather from least desirable to most desirable. This was corrected for in the analysis by separating some of the test results and grading them as a + or a -, however, this reduction from a 1-4 scale to a +/- might hide some of the nuances in the material and possibly increase the chance of a type 1 error.

A selection of some remarks and their concordant search intensity averages

Score	Remark
2	*"Dog for donation/No search ability"*
2	*Donation*
2,3	*"Donation! No search intensity/ability"*
2,3	*"Donation"*
2,3	*"Donation"*
3	*Most likely donation dog*
3,67	*"Could be OK"*
4	*"Good!! Breeding!!"*
4	*"Very good dog!!"*
4	*"Breeding"*
4	*"Breeding!!"*
4	*"Very Good!!..."*
4	*"Very good!!..."*
4	*"Good Dog, breeding? Truls male"*

Table 37

For the interpretation of the results, it is also worth reminding that we have not done a correction for multiple testing. Therefore, we may have overestimated the number of significant associations. It is, however, also important to remember that the tested candidate genes are picked out due to references indicating that such associations exist and that most of the recorded phenotypes contain some anxiety components that may have a common genetic/etiological background. It is also interesting to note that despite the limitation of this study, several of the detected association to anxiety-like phenotypes point at HTR2A and one specific marker-allele.

Alberghina, D., Rizzo, M., Piccione, G., Giannetto, C. & Panzera, M. (2017). An exploratory study about the association between serum serotonin concentrations and canine-human social interactions in shelter dogs (Canis familiaris). *Journal of Veterinary Behavior*, 18: 96-101. doi: https://doi.org/10.1016/j.jveb.2016.09.006.

Allis, C. D. & Jenuwein, T. (2016). The molecular hallmarks of epigenetic control. *Nature Reviews Genetics*, 17: 487. doi: 10.1038/nrg.2016.59.

Andrews, G., Stewart, G., Allen, R. & Henderson, A. S. (1990). The genetics of six neurotic disorders: a twin study. *J Affect Disord*, 19 (1): 23-9.

APA. (2013). *Diagnostic and statistical manual of mental disorders (DSM-5®)*: American Psychiatric Pub.

Axelsson, E., Ratnakumar, A., Arendt, M. L., Maqbool, K., Webster, M. T., Perloski, M., Liberg, O., Arnemo, J. M., Hedhammar, A. & Lindblad-Toh, K. (2013). The genomic signature of dog domestication reveals adaptation to a starch-rich diet. *Nature*, 495 (7441): 360-4. doi: 10.1038/nature11837.

B., K. & J., P. (1997). Effects of Serotonin Depletion on the Play of Juvenile Rats. *Annals of the New York Academy of Sciences*, 807 (1): 475-477. doi: doi:10.1111/j.1749-6632.1997.tb51942.x.

Bae, H. T., Sebastiani, P., Sun, J. X., Andersen, S. L., Daw, E. W., Terracciano, A., Ferrucci, L. & Perls, T. T. (2013). Genome-wide association study of personality traits in the long life family study. *Front Genet*, 4: 65. doi: 10.3389/fgene.2013.00065.

Bellamy, K. (2015). *A study of candidate genes for anxiety in the Havanese breed*. Master: Norwegian University for life sciences.

Bellamy, K. K. L., Storengen, L. M., Handegård, K. W., Arnet, E. F., Prestrud, K. W., Overall, K. L. & Lingaas, F. (2018). DRD2 is associated with fear in some dog breeds. *Journal of Veterinary Behavior*, 27: 67-73. doi: https://doi.org/10.1016/j.jveb.2018.07.008.

Benecke, N. (1987). Studies on early dog remains from Northern Europe. *Journal of Archaeological Science*, 14 (1): 31-49. doi: https://doi.org/10.1016/S0305-4403(87)80004-3.

Berman, R. M., Sanacora, G., Anand, A., Roach, L. M., Fasula, M. K., Finkelstein, C. O., Wachen, R. M., Oren, D. A., Heninger, G. R. & Charney, D. S. (2002). Monoamine depletion in unmedicated depressed subjects. *Biol Psychiatry*, 51 (6): 469-73.

Boake, C. (2002). From the Binet–Simon to the Wechsler–Bellevue: Tracing the History of Intelligence Testing. *Journal of Clinical and Experimental Neuropsychology*, 24 (3): 383-405. doi: 10.1076/jcen.24.3.383.981.

Boardman, J. D. (2009). State-level moderation of genetic tendencies to smoke. *Am J Public Health*, 99 (3): 480-6. doi: 10.2105/ajph.2008.134932.

Bortolato, M., Godar, S. C., Tambaro, S., Li, F. G., Devoto, P., Coba, M. P., Chen, K. & Shih, J. C. (2013). Early postnatal inhibition of serotonin synthesis results in long-term reductions of perseverative behaviors, but not aggression, in MAO A-deficient mice. *Neuropharmacology*, 75: 223-232. doi: https://doi.org/10.1016/j.neuropharm.2013.07.003.

Botigué, L. R., Song, S., Scheu, A., Gopalan, S., Pendleton, A. L., Oetjens, M., Taravella, A. M., Seregély, T., Zeeb-Lanz, A., Arbogast, R.-M., et al. (2017). Ancient European dog genomes reveal continuity since the Early Neolithic. *Nature Communications*, 8: 16082. doi: 10.1038/ncomms16082

https://www.nature.com/articles/ncomms16082#supplementary-information.

Bouchard, T. J., Jr. & McGue, M. (2003). Genetic and environmental influences on human psychological differences. *J Neurobiol*, 54 (1): 4-45. doi: 10.1002/neu.10160.

Brenøe, U. T., Larsgard, A. G., Johannessen, K.-R. & Uldal, S. H. (2002). Estimates of genetic parameters for hunting performance traits in three breeds of gun hunting dogs in Norway. *Applied Animal Behaviour Science*, 77 (3): 209-215. doi: https://doi.org/10.1016/S0168-1591(02)00050-3.

Bunney, W. E., Jr & Davis, J. M. (1965). Norepinephrine in depressive reactions: A review. *Archives of General Psychiatry*, 13 (6): 483-494. doi: 10.1001/archpsyc.1965.01730060001001.

Bönsch, D., Lenz, B., Reulbach, U., Kornhuber, J. & Bleich, S. (2004). Homocysteine associated genomic DNA hypermethylation in patients with chronic alcoholism. *Journal of Neural Transmission*, 111 (12): 1611-1616. doi: 10.1007/s00702-004-0232-x.

Caspi, A., Sugden, K., Moffitt, T. E., Taylor, A., Craig, I. W., Harrington, H., McClay, J., Mill, J., Martin, J., Braithwaite, A., et al. (2003). Influence of Life Stress on Depression: Moderation by a Polymorphism in the 5-HTT Gene. *Science*, 301 (5631): 386-389. doi: 10.1126/science.1083968.

Chipuer, H. M., Rovine, M. J. & Plomin, R. (1990). LISREL modeling: Genetic and environmental influences on IQ revisited. *Intelligence*, 14 (1): 11-29. doi: https://doi.org/10.1016/0160-2896(90)90011-H.

Chou, I. J., Kuo, C.-F., Huang, Y.-S., Grainge, M. J., Valdes, A. M., See, L.-C., Yu, K.-H., Luo, S.-F., Huang, L.-S., Tseng, W.-Y., et al. (2017). Familial Aggregation and Heritability of Schizophrenia and Co-aggregation of Psychiatric Illnesses in Affected Families. *Schizophrenia Bulletin*, 43 (5): 1070-1078. doi: 10.1093/schbul/sbw159.

Colombo, A. C., de Oliveira, A. R., Reimer, A. E. & Brandão, M. L. (2013). Dopaminergic mechanisms underlying catalepsy, fear and anxiety: Do they interact? *Behavioural Brain Research*, 257: 201-207. doi: https://doi.org/10.1016/j.bbr.2013.10.002.

Curt Blixt, P. A., Kenth Svartberg, Sophia Trenkle Nyberg. (2010). Utarbetande av ett beteende- och personlighetstest för avelsändamål. *Svenska Kennelklubben*.

Davis, R. N. (1999). Web-based administration of a personality questionnaire: Comparison with traditional methods. *Behavior Research Methods, Instruments, & Computers*, 31 (4): 572-577. doi: 10.3758/bf03200737.

de Moor, M. H., Costa, P. T., Terracciano, A., Krueger, R. F., de Geus, E. J., Toshiko, T., Penninx, B. W., Esko, T., Madden, P. A., Derringer, J., et al. (2012). Meta-analysis of genome-wide association studies for personality. *Mol Psychiatry*, 17 (3): 337-49. doi: 10.1038/mp.2010.128.

Delgado, P. L. (2000). Depression: the case for a monoamine deficiency. *J Clin Psychiatry*, 61 Suppl 6: 7-11.

Diederich, C. & Giffroy, J.-M. (2006). Behavioural testing in dogs: A review of methodology in search for standardisation. *Applied Animal Behaviour Science*, 97 (1): 51-72. doi: 10.1016/j.applanim.2005.11.018.

Dodman, N. H., Karlsson, E. K., Moon-Fanelli, A., Galdzicka, M., Perloski, M., Shuster, L., Lindblad-Toh, K. & Ginns, E. I. (2009). A canine chromosome 7 locus confers compulsive disorder susceptibility. *Molecular Psychiatry*, 15: 8. doi: 10.1038/mp.2009.111

https://www.nature.com/articles/mp2009111#supplementary-information.

Duffy, D. L., Hsu, Y. & Serpell, J. A. (2008). Breed differences in canine aggression. *Applied Animal Behaviour Science*, 114 (3): 441-460. doi: 10.1016/j.applanim.2008.04.006.

Döring, D., Haberland, B. E., Bauer, A., Dobenecker, B., Hack, R., Schmidt, J. & Erhard, M. H. (2016). Behavioral observations in dogs in 4 research facilities: Do they use their enrichment? *Journal of Veterinary Behavior: Clinical Applications and Research*, 13: 55-62. doi: https://doi.org/10.1016/j.jveb.2016.04.002.

Eisenberg, D. T. A., MacKillop, J., Modi, M., Beauchemin, J., Dang, D., Lisman, S. A., Lum, J. K. & Wilson, D. S. (2007). Examining impulsivity as an endophenotype using a behavioral approach: a DRD2 TaqI A and DRD4 48-bp VNTR association study. *Behavioral and Brain Functions*, 3 (1): 2. doi: 10.1186/1744-9081-3-2.

Flint, J. & Munafò, M. R. (2007). The endophenotype concept in psychiatric genetics. *Psychological Medicine*, 37 (2): 163-180. doi: 10.1017/S0033291706008750.

Folstein, S. & Rutter, M. (1977). INFANTILE AUTISM: A GENETIC STUDY OF 21 TWIN PAIRS. *Journal of Child Psychology and Psychiatry*, 18 (4): 297-321. doi: doi:10.1111/j.1469-7610.1977.tb00443.x.

Fontaine, N. M., Rijsdijk, F. V., McCrory, E. J. & Viding, E. (2010). Etiology of different developmental trajectories of callous-unemotional traits. *J Am Acad Child Adolesc Psychiatry*, 49 (7): 656-64. doi: 10.1016/j.jaac.2010.03.014.

Fowler, J. H., Baker, L. A. & Dawes, C. T. (2008). Genetic Variation in Political Participation. *American Political Science Review*, 102 (2): 233-248. doi: 10.1017/S0003055408080209.

Frantz, L. A. F., Mullin, V. E., Pionnier-Capitan, M., Lebrasseur, O., Ollivier, M., Perri, A., Linderholm, A., Mattiangeli, V., Teasdale, M. D., Dimopoulos, E. A., et al. (2016). Genomic and archaeological evidence suggest a dual origin of domestic dogs. *Science*, 352 (6290): 1228.

Frazer, A. & Hensler, J. (1999). *Serotonin involvement in physiological function and behavior*: Lippincott-Raven, Philadelphia, Pa, USA. pp. 1-5.

Galton, F. (1869). *Hereditary genius: An inquiry into its laws and consequences*, vol. 27: Macmillan.

GeneCodes. *Sequencher* (Version 5.4.6).

Gerdts, J. & Bernier, R. (2011). The broader autism phenotype and its implications on the etiology and treatment of autism spectrum disorders. *Autism research and treatment*, 2011: 545901-545901. doi: 10.1155/2011/545901.

Godar, S. C., Bortolato, M., Frau, R., Dousti, M., Chen, K. & Shih, J. C. (2011). Maladaptive defensive behaviours in monoamine oxidase A-deficient mice. *International Journal of Neuropsychopharmacology*, 14 (9): 1195-1207. doi: 10.1017/S1461145710001483.

Goodloe, L. P. & Borchelt, P. L. (1998). Companion dog temperament traits. *J Appl Anim Welf Sci*, 1 (4): 303-38. doi: 10.1207/s15327604jaws0104_1.

Goodwin, F. & Jamison, K. (1990). Manic. *Depressive Illness. New York, NY: Oxford University Press*.

Gosling, S. D., Rentfrow, P. J. & Swann, W. B. (2003). A very brief measure of the Big-Five personality domains. *Journal of Research in Personality*, 37 (6): 504-528. doi: https://doi.org/10.1016/S0092-6566(03)00046-1.

Gottesman, II & Bertelsen, A. (1989). Confirming unexpressed genotypes for schizophrenia. Risks in the offspring of Fischer's Danish identical and fraternal discordant twins. *Arch Gen Psychiatry*, 46 (10): 867-72.

Gottesman, I. I. (1990). *Schizophrenia Genesis: The Origins of Madness*: Henry Holt and Company.

Guy, N. C., Luescher, U. A., Dohoo, S. E., Spangler, E., Miller, J. B., Dohoo, I. R. & Bate, L. A. (2001). Risk factors for dog bites to owners in a general veterinary caseload. *Applied Animal Behaviour Science*, 74 (1): 29-42. doi: https://doi.org/10.1016/S0168-1591(01)00154-X.

Hall, N. J. & Wynne, C. D. L. (2012). The canid genome: Behavioral geneticists' best friend? *Genes, Brain and Behavior*, 11 (8): 889-902. doi: 10.1111/j.1601-183X.2012.00851.x.

Hanin, I. (1978). Central neurotransmitter function and its behavioral correlates in man. *Environmental Health Perspectives*, 26: 135-141.

Hart, B. L. & Miller, M. F. (1985). Behavioral profiles of dog breeds. *J Am Vet Med Assoc*, 186 (11): 1175-80.

Haworth, C. M., Wright, M. J., Luciano, M., Martin, N. G., de Geus, E. J., van Beijsterveldt, C. E., Bartels, M., Posthuma, D., Boomsma, D. I., Davis, O. S., et al. (2010). The heritability of general cognitive ability increases linearly from childhood to young adulthood. *Mol Psychiatry*, 15 (11): 1112-20. doi: 10.1038/mp.2009.55.

Henderson, N. D., Turri, M. G., DeFries, J. C. & Flint, J. (2004). QTL analysis of multiple behavioral measures of anxiety in mice. *Behav Genet*, 34 (3): 267-93. doi: 10.1023/b:Bege.0000017872.25069.44.

Hettema, J. M., Neale, M. C. & Kendler, K. S. (2001). A review and meta-analysis of the genetic epidemiology of anxiety disorders. *Am J Psychiatry*, 158 (10): 1568-78. doi: 10.1176/appi.ajp.158.10.1568.

Hirschfeld, R. M. A. (2001). The Comorbidity of Major Depression and Anxiety Disorders: Recognition and Management in Primary Care. *Primary Care Companion to The Journal of Clinical Psychiatry*, 3 (6): 244-254.

Holliday, R. & Ho, T. (2002). DNA methylation and epigenetic inheritance. *Methods*, 27 (2): 179-83.

Holmes, A., Kinney, J. W., Wrenn, C. C., Li, Q., Yang, R. J., Ma, L., Vishwanath, J., Saavedra, M. C., Innerfield, C. E., Jacoby, A. S., et al. (2003). Galanin GAL-R1 receptor null mutant mice display increased anxiety-like behavior specific to the elevated plus-maze. *Neuropsychopharmacology*, 28 (6): 1031-44. doi: 10.1038/sj.npp.1300164.

Homberg, J. R., Schiepers, O. J. G., Schoffelmeer, A. N. M., Cuppen, E. & Vanderschuren, L. J. M. J. (2007). Acute and constitutive increases in central serotonin levels reduce social play behaviour in peri-adolescent rats. *Psychopharmacology*, 195 (2): 175. doi: 10.1007/s00213-007-0895-8.

Houpt, K. A. (2007). Genetics of canine behavior. *Acta Veterinaria Brno*, 76 (3): 431-444. doi: 10.2754/avb200776030431.

Hradecká, L., Bartoš, L., Svobodová, I. & Sales, J. (2015). Heritability of behavioural traits in domestic dogs: A meta-analysis. *Applied Animal Behaviour Science*, 170: 1-13. doi: https://doi.org/10.1016/j.applanim.2015.06.006.

Hsu, Y. & Serpell, J. (2003). *Development and validation of a questionnaire for measuring behavior and temperament traits in pet dogs*, vol. 223.

Hurley, R. S., Losh, M., Parlier, M., Reznick, J. S. & Piven, J. (2007). The broad autism phenotype questionnaire. *J Autism Dev Disord*, 37 (9): 1679-90. doi: 10.1007/s10803-006-0299-3.

Jang, K. L., Livesley, W. J. & Vernon, P. A. (1996). Heritability of the big five personality dimensions and their facets: a twin study. *J Pers*, 64 (3): 577-91.

Jensen, P. (2013). Transgenerational epigenetic effects on animal behaviour. *Prog Biophys Mol Biol*, 113 (3): 447-54. doi: 10.1016/j.pbiomolbio.2013.01.001.

Kaminsky, Z., Petronis, A., Wang, S.-C., Levine, B., Ghaffar, O., Floden, D. & Feinstein, A. (2008). Epigenetics of Personality Traits: An Illustrative Study of Identical Twins Discordant for Risk-Taking Behavior. *Twin Research and Human Genetics*, 11 (1): 1-11. doi: 10.1375/twin.11.1.1.

Kanner, L. (1943). Autistic Disturbances of affective contact.

Kapczinski, F., Lima, M. S., Souza, J. S. & Schmitt, R. (2003). Antidepressants for generalized anxiety disorder. *Cochrane Database Syst Rev* (2): Cd003592. doi: 10.1002/14651858.Cd003592.

Kasper, S. (2006). Anxiety disorders: under-diagnosed and insufficiently treated. *Int J Psychiatry Clin Pract*, 10 Suppl 1: 3-9. doi: 10.1080/13651500600552297.

Katzelnick, D. J., Kobak, K. A., DeLeire, T., Henk, H. J., Greist, J. H., Davidson, J. R., Schneier, F. R., Stein, M. B. & Helstad, C. P. (2001). Impact of generalized social anxiety disorder in managed care. *American Journal of Psychiatry*, 158 (12): 1999-2007.

Kaulfuß, P. & Mills, D. S. (2008). Neophilia in domestic dogs (Canis familiaris) and its implication for studies of dog cognition. *Animal Cognition*, 11 (3): 553-556. doi: 10.1007/s10071-007-0128-x.

Kendler, K. S., Gatz, M., Gardner, C. O. & Pedersen, N. L. (2006). Personality and major depression: a Swedish longitudinal, population-based twin study. *Arch Gen Psychiatry*, 63 (10): 1113-20. doi: 10.1001/archpsyc.63.10.1113.

Kendler, K. S. & Baker, J. H. (2007). Genetic influences on measures of the environment: a systematic review. *Psychol Med*, 37 (5): 615-26. doi: 10.1017/s0033291706009524.

Kendler, K. S., Gardner, C. & Dick, D. M. (2011). Predicting alcohol consumption in adolescence from alcohol-specific and general externalizing genetic risk factors, key environmental exposures and their interaction. *Psychol Med*, 41 (7): 1507-16. doi: 10.1017/s003329171000190x.

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R. & Walters, E. E. (2005). Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry*, 62 (6): 593-602. doi: 10.1001/archpsyc.62.6.593.

Kessler, R. C. (2008). *National Comorbidity Survey: Baseline (NCS-1), 1990-1992*: Inter-university Consortium for Political and Social Research [distributor].

Lee, S. H., Wray, N. R., Goddard, M. E. & Visscher, P. M. (2011). Estimating missing heritability for disease from genome-wide association studies. *Am J Hum Genet*, 88 (3): 294-305. doi: 10.1016/j.ajhg.2011.02.002.

Liinamo, A.-E., van den Berg, L., Leegwater, P. A. J., Schilder, M. B. H., van Arendonk, J. A. M. & van Oost, B. A. (2007). Genetic variation in aggression-related traits in Golden Retriever dogs.

Applied Animal Behaviour Science, 104 (1): 95-106. doi: https://doi.org/10.1016/j.applanim.2006.04.025.
Lit, L., Belanger, J. M., Boehm, D., Lybarger, N. & Oberbauer, A. M. (2013). Differences in behavior and activity associated with a poly (a) expansion in the dopamine transporter in Belgian Malinois. *PloS one*, 8 (12): e82948.
Locke, J. (1689). *An Essay Concerning Human Understanding*, vol. 3: Oxford University Press.
Losh, M., Childress, D., Lam, K. & Piven, J. (2008). Defining key features of the broad autism phenotype: A comparison across parents of multiple- and single-incidence autism families. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, 147B (4): 424-433. doi: doi:10.1002/ajmg.b.30612.
M stephens, P. S., Nicholas Smith, Peter Donnelly. (2005). *PHASE* (Version 2.1.1).
Mackenzie, S. A., Oltenacu, E. A. B. & Houpt, K. A. (1986). Canine behavioral genetics — A review. *Applied Animal Behaviour Science*, 15 (4): 365-393. doi: https://doi.org/10.1016/0168-1591(86)90128-0.
Marston, L. C., Bennett, P. C. & Coleman, G. J. (2004). What happens to shelter dogs? An analysis of data for 1 year from three Australian shelters. *J Appl Anim Welf Sci*, 7 (1): 27-47. doi: 10.1207/s15327604jaws0701_2.
Mary, D. S. A. & Bell, S. M. (1970). Attachment, Exploration, and Separation: Illustrated by the Behavior of One-Year-Olds in a Strange Situation. *Child Development*, 41 (1): 49-67. doi: 10.2307/1127388.
Masterpasqua, F. (2009). *Psychology and epigenetics*, 13. US: Educational Publishing Foundation. pp. 194-201.
McCune, S. (1995). The impact of paternity and early socialisation on the development of cats' behaviour to people and novel objects. *Applied Animal Behaviour Science*, 45 (1-2): 109-124. doi: 10.1016/0168-1591(95)00603-P.
McDonald, J. H. (2009). *Handbook of biological statistics*, vol. 2: Sparky House Publishing Baltimore, MD.
McEwen, B. S. (1998). Protective and damaging effects of stress mediators. *N Engl J Med*, 338 (3): 171-9. doi: 10.1056/nejm199801153380307.
McGuffin, P., Farmer, A. & Gottesman, I. I. (1987). Is There Really A Split in Schizophrenia?: The Genetic Evidence. *British Journal of Psychiatry*, 150 (5): 581-592. doi: 10.1192/bjp.150.5.581.
McGuffin, P., Katz, R. & Bebbington, P. (1987). Hazard, heredity and depression. A family study. *Journal of Psychiatric Research*, 21 (4): 365-375. doi: https://doi.org/10.1016/0022-3956(87)90083-5.
McGuffin, P., Katz, R., Watkins, S. & Rutherford, J. (1996). A hospital-based twin register of the heritability of DSM-IV unipolar depression. *Arch Gen Psychiatry*, 53 (2): 129-36.
McGuffin, P., Rijsdijk, F., Andrew, M., Sham, P., Katz, R. & Cardno, A. (2003). The heritability of bipolar affective disorder and the genetic relationship to unipolar depression. *Arch Gen Psychiatry*, 60 (5): 497-502. doi: 10.1001/archpsyc.60.5.497.
Mesulam, M.-M. (2000). *Principles of behavioral and cognitive neurology*: Oxford University Press.
Miller, G. (2010). The Seductive Allure of Behavioral Epigenetics. *Science*, 329 (5987): 24.
Mondelli, F., Prato Previde, E., Verga, M., Levi, D., Magistrelli, S. & Valsecchi, P. (2004). The Bond That Never Developed: Adoption and Relinquishment of Dogs in a Rescue Shelter. *Journal of Applied Animal Welfare Science*, 7 (4): 253-266. doi: 10.1207/s15327604jaws0704_3.
Morisano, D., Babor, T. F. & Robaina, K. A. (2014). Co-Occurrence of Substance use Disorders with other Psychiatric Disorders: Implications for Treatment Services. *Nordic Studies on Alcohol and Drugs*, 31 (1): 5-25. doi: 10.2478/nsad-2014-0002.
Mosing, M. A., Gordon, S. D., Medland, S. E., Statham, D. J., Nelson, E. C., Heath, A. C., Martin, N. G. & Wray, N. R. (2009). Genetic and environmental influences on the co-morbidity between depression, panic disorder, agoraphobia, and social phobia: a twin study. *Depress Anxiety*, 26 (11): 1004-11. doi: 10.1002/da.20611.

Munafo, M. R. & Flint, J. (2004). Meta-analysis of genetic association studies. *Trends Genet*, 20 (9): 439-44. doi: 10.1016/j.tig.2004.06.014.

Munafò, M. R. (2006). Candidate gene studies in the 21st century: meta-analysis, mediation, moderation. *Genes, Brain and Behavior*, 5 (S1): 3-8. doi: 10.1111/j.1601-183X.2006.00188.x.

Overall, K. L., Dunham, A. E. & Frank, D. (2001). Frequency of nonspecific clinical signs in dogs with separation anxiety, thunderstorm phobia, and noise phobia, alone or in combination. *J Am Vet Med Assoc*, 219 (4): 467-73.

Overall, K. L., Hamilton, S. P. & Chang, M. L. (2006). Understanding the genetic basis of canine anxiety: phenotyping dogs for behavioral, neurochemical, and genetic assessment. *Journal of Veterinary Behavior: Clinical Applications and Research*, 1 (3): 124-141. doi: 10.1016/j.jveb.2006.09.004.

Packer, R., Hendricks, A. & Burn, C. (2012). Do dog owners perceive the clinical signs related to conformational inherited disorders as' normal'for the breed? A potential constraint to improving canine welfare. *Animal Welfare-The UFAW Journal*, 21 (1): 81.

Pak, S. (2004). The biological standard of living in the two Koreas. *Econ Hum Biol*, 2 (3): 511-21. doi: 10.1016/j.ehb.2004.09.001.

Patel, D. R., Feucht, C., Brown, K. & Ramsay, J. (2018). Pharmacological treatment of anxiety disorders in children and adolescents: a review for practitioners. *Transl Pediatr*, 7 (1): 23-35. doi: 10.21037/tp.2017.08.05.

Peremans, K., Audenaert, K., Coopman, F., Blanckaert, P., Jacobs, F., Otte, A., Verschooten, F., van Bree, H., van Heeringen, K., Mertens, J., et al. (2003). Estimates of regional cerebral blood flow and 5-HT2A receptor density in impulsive, aggressive dogs with 99mTc-ECD and 123I-5-I-R91150. *Eur J Nucl Med Mol Imaging*, 30 (11): 1538-46. doi: 10.1007/s00259-003-1250-x.

Perneger, T. V. (1998). What's wrong with Bonferroni adjustments. *BMJ : British Medical Journal*, 316 (7139): 1236-1238.

Persson, M. E., Roth, L. S. V., Johnsson, M., Wright, D. & Jensen, P. (2015). Human-directed social behaviour in dogs shows significant heritability. *Genes, Brain and Behavior*, 14 (4): 337-344. doi: doi:10.1111/gbb.12194.

Plomin, R., DeFries, J. C., Knopik, V. S. & Neiderhiser, J. M. (2012). *Behavioral Genetics*: Worth Publishers.

Pogue-Geile, M., Ferrell, R., Deka, R., Debski, T. & Manuck, S. (1998). Human novelty-seeking personality traits and dopamine D4 receptor polymorphisms: a twin and genetic association study. *American Journal of Medical Genetics*, 81 (1): 44-48. doi: doi:10.1002/(SICI)1096-8628(19980207)81:1<44::AID-AJMG9>3.0.CO;2-N.

Popova, N. K., Kulikov, A. V., Avgustinovich, D. F., Voitenko, N. N. & Trut, L. N. (1997). [Effect of domestication of the silver fox on the main enzymes of serotonin metabolism and serotonin receptors]. *Genetika*, 33 (3): 370-4.

Power, R. A. & Pluess, M. (2015a). Heritability estimates of the Big Five personality traits based on common genetic variants. *Translational psychiatry*, 5 (7): e604-e604. doi: 10.1038/tp.2015.96.

Power, R. A. & Pluess, M. (2015b). Heritability estimates of the Big Five personality traits based on common genetic variants. *Translational Psychiatry*, 5: e604. doi: 10.1038/tp.2015.96.

Proschowsky, H. F., Rugbjerg, H. & Ersboll, A. K. (2003). Mortality of purebred and mixed-breed dogs in Denmark. *Preventive Veterinary Medicine*, 58 (1-2): 63-74. doi: 10.1016/s0167-5877(03)00010-2.

Ptáček, R., Kuželová, H. & Stefano, G. B. (2011). Dopamine D4 receptor gene DRD4 and its association with psychiatric disorders. *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research*, 17 (9): RA215-RA220. doi: 10.12659/MSM.881925.

Purcell, S. M., Wray, N. R., Stone, J. L., Visscher, P. M., O'Donovan, M. C., Sullivan, P. F. & Sklar, P. (2009). Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. *Nature*, 460 (7256): 748-52. doi: 10.1038/nature08185.

Puurunen, J., Sulkama, S., Tiira, K., Araujo, C., Lehtonen, M., Hanhineva, K. & Lohi, H. (2016). A non-targeted metabolite profiling pilot study suggests that tryptophan and lipid metabolisms are linked with ADHD-like behaviours in dogs. *Behav Brain Funct*, 12 (1): 27. doi: 10.1186/s12993-016-0112-1.

Reuterwall, C. & Ryman, N. (1973). An estimate of the magnitude of additive genetic variation of some mental characters in Alsatian dogs. *Hereditas*, 73 (2): 277-283. doi: 10.1111/j.1601-5223.1973.tb01090.x.

Rietveld, C. A., Conley, D., Eriksson, N., Esko, T., Medland, S. E., Vinkhuyzen, A. A. E., Yang, J., Boardman, J. D., Chabris, C. F., Dawes, C. T., et al. (2014). Replicability and robustness of genome-wide-association studies for behavioral traits. *Psychological science*, 25 (11): 1975-1986. doi: 10.1177/0956797614545132.

Riley, B. & Kendler, K. S. (2006). Molecular genetic studies of schizophrenia. *Eur J Hum Genet*, 14 (6): 669-80. doi: 10.1038/sj.ejhg.5201571.

Ritsner, M. & Gottesman, I. (2011). *The Schizophrenia Construct After 100 Years of Challenges*.

Riva, J., Bondiolotti, G., Michelazzi, M., Verga, M. & Carenzi, C. (2008). Anxiety related behavioural disorders and neurotransmitters in dogs. *Applied Animal Behaviour Science*, 114 (1): 168-181. doi: https://doi.org/10.1016/j.applanim.2008.01.020.

Rooney, N. J., Bradshaw, J. W. S. & Robinson, I. H. (2000). A comparison of dog–dog and dog–human play behaviour. *Applied Animal Behaviour Science*, 66 (3): 235-248. doi: https://doi.org/10.1016/S0168-1591(99)00078-7.

Saetre, P., Strandberg, E., Sundgren, P. E., Pettersson, U., Jazin, E. & Bergström, T. F. (2006). The genetic contribution to canine personality. *Genes, Brain and Behavior*, 5 (3): 240-248. doi: doi:10.1111/j.1601-183X.2005.00155.x.

Saha, S., Chant, D., Welham, J. & McGrath, J. (2005). A systematic review of the prevalence of schizophrenia. *PLoS Med*, 2 (5): e141. doi: 10.1371/journal.pmed.0020141.

Salman, M. D., New, J. G., Jr., Scarlett, J. M., Kass, P. H., Ruch-Gallie, R. & Hetts, S. (1998). Human and animal factors related to relinquishment of dogs and cats in 12 selected animal shelters in the United States. *J Appl Anim Welf Sci*, 1 (3): 207-26. doi: 10.1207/s15327604jaws0103_2.

Salomon, R. M., Miller, H. L., Krystal, J. H., Heninger, G. R. & Charney, D. S. (1997). Lack of behavioral effects of monoamine depletion in healthy subjects. *Biol Psychiatry*, 41 (1): 58-64.

Sarviaho, R., Hakosalo, O., Tiira, K., Sulkama, S., Salmela, E., Hytonen, M. K., Sillanpaa, M. J. & Lohi, H. (2019). Two novel genomic regions associated with fearfulness in dogs overlap human neuropsychiatric loci. *Transl Psychiatry*, 9 (1): 18. doi: 10.1038/s41398-018-0361-x.

Schwekendiek, D. (2009). HEIGHT AND WEIGHT DIFFERENCES BETWEEN NORTH AND SOUTH KOREA. *Journal of Biosocial Science*, 41 (1): 51-55. doi: 10.1017/S002193200800299X.

Scott, J. P. & Fuller, J. L. (1974). *Genetics and the Social Behavior of the Dog*: University of Chicago Press.

Segman, R. H., Cooper-Kazaz, R., Macciardi, F., Goltser, T., Halfon, Y., Dobroborski, T. & Shalev, A. Y. (2002). Association between the dopamine transporter gene and posttraumatic stress disorder. *Molecular Psychiatry*, 7: 903. doi: 10.1038/sj.mp.4001085.

Senaratne, R., Van Ameringen, M., Mancini, C. & Patterson, B. (2010). The burden of anxiety disorders on the family. *J Nerv Ment Dis*, 198 (12): 876-80. doi: 10.1097/NMD.0b013e3181fe7450.

Serpell, J. A. (1996). Evidence for an association between pet behavior and owner attachment levels. *Applied Animal Behaviour Science*, 47 (1): 49-60. doi: https://doi.org/10.1016/0168-1591(95)01010-6.

Serpell, J. A. & Hsu, Y. (2001). Development and validation of a novel method for evaluating behavior and temperament in guide dogs. *Appl Anim Behav Sci*, 72 (4): 347-364.

Siviy, S. M., Deron, L. M. & Kasten, C. R. (2011). Serotonin, motivation, and playfulness in the juvenile rat. *Developmental Cognitive Neuroscience*, 1 (4): 606-616. doi: https://doi.org/10.1016/j.dcn.2011.07.002.

Solanki, R. K., Singh, P., Midha, A. & Chugh, K. (2008). Schizophrenia: Impact on quality of life. *Indian Journal of Psychiatry*, 50 (3): 181-186. doi: 10.4103/0019-5545.43632.
Soomro, G. M., Altman, D., Rajagopal, S. & Oakley-Browne, M. (2008). Selective serotonin re-uptake inhibitors (SSRIs) versus placebo for obsessive compulsive disorder (OCD). *Cochrane Database Syst Rev* (1): Cd001765. doi: 10.1002/14651858.CD001765.pub3.
Staats, S., Wallace, H. & Anderson, T. (2008). Reasons for companion animal guardianship (pet ownership) from two populations. *Society & Animals*, 16 (3): 279-291.
Steel, Z., Marnane, C., Iranpour, C., Chey, T., Jackson, J. W., Patel, V. & Silove, D. (2014). The global prevalence of common mental disorders: a systematic review and meta-analysis 1980-2013. *International journal of epidemiology*, 43 (2): 476-493. doi: 10.1093/ije/dyu038.
Stein, M. B., Jang, K. L. & Livesley, W. J. (1999). Heritability of anxiety sensitivity: a twin study. *Am J Psychiatry*, 156 (2): 246-51. doi: 10.1176/ajp.156.2.246.
Stephan Ripke. (2014). Biological insights from 108 schizophrenia-associated genetic loci. *Nature*, 511 (7510): 421-7. doi: 10.1038/nature13595.
Storengen, L. M. (2015). *Genetic Studies of canine anxiety*: Norwegian University of life sciences.
Storengen, L. M. & Lingaas, F. (2015). Noise sensitivity in 17 dog breeds: Prevalence, breed risk and correlation with fear in other situations. *Applied Animal Behaviour Science*, 171: 152-160. doi: https://doi.org/10.1016/j.applanim.2015.08.020.
Sullivan, P. F., Neale, M. C. & Kendler, K. S. (2000). Genetic epidemiology of major depression: review and meta-analysis. *Am J Psychiatry*, 157 (10): 1552-62. doi: 10.1176/appi.ajp.157.10.1552.
Sullivan, P. F., Kendler, K. S. & Neale, M. C. (2003). Schizophrenia as a complex trait: evidence from a meta-analysis of twin studies. *Arch Gen Psychiatry*, 60 (12): 1187-92. doi: 10.1001/archpsyc.60.12.1187.
Sutter, N. B., Eberle, M. A., Parker, H. G., Pullar, B. J., Kirkness, E. F., Kruglyak, L. & Ostrander, E. A. (2004). Extensive and breed-specific linkage disequilibrium in Canis familiaris. *Genome Res*, 14 (12): 2388-96. doi: 10.1101/gr.3147604.
Svartberg, K. (2005). A comparison of behaviour in test and in everyday life: evidence of three consistent boldness-related personality traits in dogs. *Applied Animal Behaviour Science*, 91 (1): 103-128. doi: https://doi.org/10.1016/j.applanim.2004.08.030.
Tambs, K., Czajkowsky, N., R⊘ysamb, E., Neale, M. C., Reichborn-Kjennerud, T., Aggen, S. H., Harris, J. R., ⊘rstavik, R. E. & Kendler, K. S. (2009). Structure of genetic and environmental risk factors for dimensional representations of DSM–IV anxiety disorders. *British Journal of Psychiatry*, 195 (4): 301-307. doi: 10.1192/bjp.bp.108.059485.
Tandon, R., Gaebel, W., Barch, D. M., Bustillo, J., Gur, R. E., Heckers, S., Malaspina, D., Owen, M. J., Schultz, S., Tsuang, M., et al. (2013). Definition and description of schizophrenia in the DSM-5. *Schizophr Res*, 150 (1): 3-10. doi: 10.1016/j.schres.2013.05.028.
Terracciano, A., Sanna, S., Uda, M., Deiana, B., Usala, G., Busonero, F., Maschio, A., Scally, M., Patriciu, N., Chen, W. M., et al. (2010). Genome-wide association scan for five major dimensions of personality. *Mol Psychiatry*, 15 (6): 647-56. doi: 10.1038/mp.2008.113.
Tiira, K., Escriou, C., Thomas, A., Renier, S., de Citres, C. D., Koskinen, L., Kareinen, L., Arnold, P. & Lohi, H. (2011). Phenotypic and genetic characterization of tail chasing in bull terriers. *Journal of Veterinary Behavior: Clinical Applications and Research*, 6 (1): 83. doi: 10.1016/j.jveb.2010.09.005.
Tiira, K., Sulkama, S. & Lohi, H. (2016). Prevalence, comorbidity, and behavioral variation in canine anxiety. *Journal of Veterinary Behavior*, 16: 36-44. doi: https://doi.org/10.1016/j.jveb.2016.06.008.
Tran, L., Lasher, B. K., Young, K. A. & Keele, N. B. (2013). Depletion of serotonin in the basolateral amygdala elevates glutamate receptors and facilitates fear-potentiated startle. *Translational Psychiatry*, 3: e298. doi: 10.1038/tp.2013.66

https://www.nature.com/articles/tp201366#supplementary-information.

Trudeau, L.-É. (2004). Glutamate co-transmission as an emerging concept in monoamine neuron function. *Journal of Psychiatry and Neuroscience*, 29 (4): 296-310.
Trut, L. N. (1999). Early Canid Domestication: The Farm-Fox Experiment: Foxes bred for tamability in a 40-year experiment exhibit remarkable transformations that suggest an interplay between behavioral genetics and development. *American Scientist*, 87 (2): 160-169.
Turkheimer, E. (2000). Three Laws of Behavior Genetics and What They Mean. *Current Directions in Psychological Science*, 9 (5): 160-164. doi: 10.1111/1467-8721.00084.
Turkheimer, E., Haley, A., Waldron, M., D'Onofrio, B. & Gottesman, II. (2003). Socioeconomic status modifies heritability of IQ in young children. *Psychol Sci*, 14 (6): 623-8. doi: 10.1046/j.0956-7976.2003.psci_1475.x.
Turner, D. C., Feaver, J., Mendl, M. & Bateson, P. (1986). Variation in domestic cat behaviour towards humans: a paternal effect. *Animal Behaviour*, 34 (6): 1890-1892. doi: https://doi.org/10.1016/S0003-3472(86)80275-5.
Valenstein, E. S. (2002). The Discovery of Chemical Neurotransmitters. *Brain and Cognition*, 49 (1): 73-95. doi: https://doi.org/10.1006/brcg.2001.1487.
van den Berg, L., Schilder, M. B. H. & Knol, B. W. (2003). Behavior Genetics of Canine Aggression: Behavioral Phenotyping of Golden Retrievers by Means of an Aggression Test. *Behavior Genetics*, 33 (5): 469-483. doi: 10.1023/a:1025714431089.
van den Berg, L., Kwant, L., Hestand, M. S., van Oost, B. A. & Leegwater, P. A. (2005). Structure and variation of three canine genes involved in serotonin binding and transport: the serotonin receptor 1A gene (htr1A), serotonin receptor 2A gene (htr2A), and serotonin transporter gene (slc6A4). *Journal of Heredity*, 96 (7): 786-796.
van den Berg, L., Schilder, M. B., de Vries, H., Leegwater, P. A. & van Oost, B. A. (2006). Phenotyping of aggressive behavior in golden retriever dogs with a questionnaire. *Behav Genet*, 36 (6): 882-902. doi: 10.1007/s10519-006-9089-0.
van Rooy, D., Arnott, E. R., Early, J. B., McGreevy, P. & Wade, C. M. (2014). Holding back the genes: limitations of research into canine behavioural genetics. *Canine Genetics and Epidemiology*, 1: 7. doi: 10.1186/2052-6687-1-7.
Verhulst, B., Neale, M. C. & Kendler, K. S. (2015). The heritability of alcohol use disorders: a meta-analysis of twin and adoption studies. *Psychol Med*, 45 (5): 1061-72. doi: 10.1017/s0033291714002165.
Vermeire, S. T., Audenaert, K. R., Dobbeleir, A. A., De Meester, R. H., De Vos, F. J. & Peremans, K. Y. (2009). Evaluation of the brain 5-HT2A receptor binding index in dogs with anxiety disorders, measured with 123I-5I-R91150 and SPECT. *J Nucl Med*, 50 (2): 284-9. doi: 10.2967/jnumed.108.055731.
Vinkhuyzen, A. A., Pedersen, N. L., Yang, J., Lee, S. H., Magnusson, P. K., Iacono, W. G., McGue, M., Madden, P. A., Heath, A. C., Luciano, M., et al. (2012). Common SNPs explain some of the variation in the personality dimensions of neuroticism and extraversion. *Transl Psychiatry*, 2: e102. doi: 10.1038/tp.2012.27.
vonHoldt, B. M., Shuldiner, E., Koch, I. J., Kartzinel, R. Y., Hogan, A., Brubaker, L., Wanser, S., Stahler, D., Wynne, C. D. L., Ostrander, E. A., et al. (2017). Structural variants in genes associated with human Williams-Beuren syndrome underlie stereotypical hypersociability in domestic dogs. *Science Advances*, 3 (7).
Walters, G. D. (2002). The heritability of alcohol abuse and dependence: a meta-analysis of behavior genetic research. *Am J Drug Alcohol Abuse*, 28 (3): 557-84.
Wan, M., Kubinyi, E., Miklósi, Á. & Champagne, F. (2009). A cross-cultural comparison of reports by German Shepherd owners in Hungary and the United States of America. *Applied Animal Behaviour Science*, 121 (3): 206-213. doi: https://doi.org/10.1016/j.applanim.2009.09.015.
Wiener, P. & Haskell, M. J. (2016). Use of questionnaire-based data to assess dog personality. *Journal of Veterinary Behavior*, 16: 81-85. doi: https://doi.org/10.1016/j.jveb.2016.10.007.
Wilsson, E. & Sundgren, P.-E. (1997). The use of a behaviour test for the selection of dogs for service and breeding, I: Method of testing and evaluating test results in the adult dog, demands on

different kinds of service dogs, sex and breed differences. *Applied Animal Behaviour Science*, 53 (4): 279-295. doi: https://doi.org/10.1016/S0168-1591(96)01174-4.

Wilsson, E. & Sundgren, P.-E. (1998). Behaviour test for eight-week old puppies—heritabilities of tested behaviour traits and its correspondence to later behaviour. *Applied Animal Behaviour Science*, 58 (1): 151-162. doi: https://doi.org/10.1016/S0168-1591(97)00093-2.

Wong, D. T., Perry, K. W. & Bymaster, F. P. (2005). Case history: the discovery of fluoxetine hydrochloride (Prozac). *Nat Rev Drug Discov*, 4 (9): 764-74. doi: 10.1038/nrd1821.

Wright, J. C. (1991). Canine aggression toward people. Bite scenarios and prevention. *Vet Clin North Am Small Anim Pract*, 21 (2): 299-314.

Zapata, I., Serpell, J. A. & Alvarez, C. E. (2016). Genetic mapping of canine fear and aggression. *BMC Genomics*, 17 (1): 572. doi: 10.1186/s12864-016-2936-3.

Zeligs, R. (1942). *Glimpses Into Child Life: The Twelve-year-old at Home and School*: W. Morrow.

Zhang, G. & Stackman, R. W. (2015). The role of serotonin 5-HT(2A) receptors in memory and cognition. *Frontiers in Pharmacology*, 6: 225. doi: 10.3389/fphar.2015.00225.

Appendix A

Description of puppy test GTC

[The following description is an unaltered copy from GTC]

Test setup.

General: The test is conducted when the puppy is 10 weeks old.

The room where the test is conducted has to be new for the puppy, it should never have entered this room before, but it may be familiar with the building. A room of approximately 10-12m^2 should be chosen that is rather empty, distracting objects such as wastepaper bins, chairs etc. should be removed prior to the test. A single chair for the tester should be placed at one end away from the wall. The tester must be a new person for the pup. Objects that are to be used for the test must be placed on a table so that they are inaccessible to the pup. The test is filmed by a person who is inaccessible for the pup (on top of a table or a freezer), there may be other observers there who are also on top of things, so they are inaccessible to the pup. None of these people may be familiar with the pup. No one speaks during the test.

Objects: a tennis ball (pups tested at GTC are not familiar with balls at all!!); a leather rug (at GTC a soft leather rug is one of the toys used for their socialisation, this is a similar one); a big thin plastic bag and a metal chain (a collar); a pen and a piece of dry pellet food.

Preparation: the tester is seated on the chair. The puppy is brought into the room by a familiar person and the door is closed.

Evaluation: the test is divided into 16 main evaluation elements that are graded observing the video registration. Each step of the test and the manner to evaluate it is described below. The grading form is designed in such a way that the most desirable behaviour is located to the right, the least desirable to the left. A simple X in the relevant box suffices. It may be that in the course of a test element, the pup improves with the repetitions (for example, a ball is thrown three times, pup first is slow to retrieve, later very quick). In case of a marked improvement, this can be coded as 1 for the beginning, and 2 for the final evaluation. For other elements that take a longer period of time, score the weakest behaviour if it lasts a significant part of the time (for example: if it takes more than 30 seconds of the 1,5 minute of the examination time of the room). In cases where a particular element was not tested, note N/A. Any observations that do not fit into the categories may be commented on in the remarks. In combination with the colour coding dividing the behaviour into environmental skills (green); hunt/retrieve behaviour (orange), prey-related behaviour (yellow) and handle ability (white) this provides a quick overview of the pups results.

Test protocol: basically the test protocol is followed in a standardised manner all the way through, except in those cases where the welfare of the pup is compromised (e.g. pup is really scared from the moment it enters the room).

Test protocol

Test element 1 & 2: room and tester

The tester sits still with hands crossed over his chest for about 1.5 min, not seeking contact with the pup. During this time the behaviour of the dog is observed with regard to its investigation of the room (1), and with regard to the tester (2).

Evaluation 1: The more free and easy the dog is in the room, the better.

- --: freezing, sitting still and whining (if this happens the test is terminated)
- -: cautiously walking around, front legs first so that the pup becomes really long, ("long Malinois") barking at things and people (if this happens the test may also be terminated, or the tester may interact a little with the pup as a "warm up")
- +: walking around looking at things
- ++: really investigating the whole room, using its nose

Evaluation 2: Relaxed, friendly contact is the best.

- --: no contact at all with the tester is not at all desirable
- -: hysterical contact, which means running towards the tester and trying for the majority of the time to get on his lap, is also not desirable
- +: contacting the tester very cautiously at first ("sneaky contact") and then coming a little closer
- ++: friendly contact, occasionally jumping up against the tester is fine

Test element 3: passive ball

The tester puts a tennis ball in front of him on the floor, taking care that it does not roll away. He times the placing of this ball in such a way that the pups attention is directed towards this ball.

Evaluation 3: Degree of ease with this new object is evaluated.

- --: the pup is afraid of this ball and does not approach it
- -: pup approaches the ball very cautiously, slightly sniffing, or shows very little interest at all
- + direct and continued interest, but just sniffing it and staying with it, maybe followed by taking it in its mouth after a while
- ++ direct interest followed by taking it in its mouth immediately

Test element 4: active ball

The tester picks up the ball and rolls it gently to one corner of the room. Depending on the behaviour of the pup, he takes it back from the pup or he goes and gets it. He repeats this a second t ime towards the same corner, and later a third time in another direction of the room.

Evaluation 4: the manner in which the pup reacts to the moving ball is evaluated. If there is a significant change in which the pup follows the ball between the 3 different throws, this can be marked by evaluating the first roll with 1 and the final roll with 2 in the relevant boxes on the form.

- --: the pup just looks at the ball rolling away and does not chase at all, maybe shows fear.
- -: the pup follows the ball a short way, not to the end.
- +: the pup follows the ball all the way to the end slowly
- ++: the pup chases the ball at high speed.

Evaluation 4A: After a + or ++ in 4, does the dog retrieve the ball? If so, evaluate:

- -: dog just smells at the ball it has followed
- +: dog picks up ball without grabbing it and brings it back, not at high speed but rather slow
- ++: dog bites the ball intensely and brings it back quickly

Evaluation 4B: After a + or ++ in 4: does the dog demonstrate prey behaviour? If so, evaluate (score the weakest behaviour!):

- --: pup defends the ball when the tester tries to take it away by biting the tester immediately
- - pup defends the ball when the tester tries to take it away by growling AND a stiff body posture
- +: pup picks up ball and runs around with it actively evading the tester, may growl but no defensive behaviour
- ++: pup picks up the ball & stays standing there, or lies down & starts chewing it, may growl but no defensive behaviour

Test element 5: disappearance ball

The tester takes the ball in his hand and makes three figures of 8 with it on the floor. The pup is usually interested in this and will follow the hand with the ball. Then the tester quickly moves his hand with the ball away from the floor behind his back, he remains seated for at least 30 seconds, or until the pup has stopped searching.

Evaluation 5: the manner in which the pup reacts to the sudden disappearance of the ball is evaluated.

- --: the pup does not search for the ball at all
- -: the pup looks around for <20 seconds or comes back to the tester for “help”
- +: the pup continues looking for it for up to 40 seconds, OR only visually, OR not really intensely
- ++: the pup continues to look for it intensely AND for longer than 40 seconds AND using its nose

Evaluation 5A: if the pup scores a ++ in 5, wait until the pup stops searching, either by changing activity or by looking at the tester repeatedly for help. When evaluating the video, calculate the time between the disappearance of the ball and the time the pup stops searching. Register this time.

Test element 6: passive rug

The tester takes a leather rug and stretches it between his hands, puts in on the floor in front of him and sits down.

Evaluation 6: degree of ease with this familiar object is observed.

- --: pup is afraid, or does not approach the rug at all
- -: pup approaches very cautiously, slightly sniffing, or shows very little interest after first approach
- +: pup is interested, sniffs it, licks or nibbles it, moves on top of it
- ++: pup is highly interested and either picks it up immediately and walks around with it, or immediately lies on top of it chewing it or guarding it

Test element 7 & 8: active rug and distraction

The tester takes the leather rug and traces it over the ground in a figure 8 whilst seated, allowing the pup to chase and if he is quick enough to catch it. He repeats this 3 times with a short interval (10-20 sec). After the third time, the tester taps with a pen on the ground about 30cm away from the rug.

Evaluation 7: the manner in which the pup reacts to the moving rug is evaluated. If there is a significant change in which the pup follows the rug between the 3 different figures of 8, this can be marked by evaluating the first chase with 1 and the final chase with 2 in the relevant boxes of the form.

- --: pup is not interested or afraid and does not chase the rug at all
- -: pup is interested and follows the movement of the rug only after a lot of movement, OR lets go of it quickly after having it is still
- +: pup is interested and follows the rug but does not really try to catch it and not at a high speed
- ++: pup follows in a high speed chase, really trying to catch it, or grabbing it

Evaluation 7A: After a + or a ++ in 7; does the pup show signs of retrieving it?

- -: the pup smells at the rug but does not pick it up in his mouth
- +: the pup takes the rug in his mouth and moves around near the tester
- ++: the pup bites the rug intensely, he may shake it, and then moves towards the tester

Evaluation 7B: After a + or a ++ is 7; does the pup show prey-related behaviour? If so, evaluate (score the weakest behaviour!):

- --: pup defends the rub when the tester tries to take it away by biting the tester immediately
- - pup defends the rug when the tester tries to take it away by growling and a stiff body posture
- +: pup stays on rug and nibbles it, or picks it up and actively evades tester, or shows demonstration behaviour showing off prey
- ++: pup stays on rug, tucking it in, and starts chewing it or takes it fully into its mouth

Evaluation 8: the manner in which the pup responds to the distraction is noted

- --: the pup growls and defends the rug, tail immobile, stiffened body posture
- -: pup is easily distracted and happily changes its attention to the pen, or has a slightly defensive body posture and maybe attacks the hand
- +: pup is not distracted at all, stays alert with waving tail but does not respond to the pen,
- ++: pup moves quickly towards the pen ("can't help himself"), but returns to the rug in a flash

Test element 9: disappearance rug

The tester stands up, and makes a 4th figure of 8 on the ground in front of him, preventing the pup from catching the rug. Then with a quick motion he pulls the rug up from the floor and hides it under his arm, and remains standing for at least 30 seconds, or until the pup has stopped searching.

Evaluation 9: the manner in which the pup reacts to the sudden disappearance of the rug is evaluated.

- --: the pup does not search for the rug at all
- -: the pup looks around for <20 seconds or comes back to the tester for "help"
- +: the pup continues looking for it for up to 40 seconds, OR only visually, OR not really intensely
- ++: the pup continues to look for it intensely AND for longer than 40 seconds AND using its nose

Evaluation 9A: if the pup scores a ++ in 5, wait until the pup stops searching, either by changing activity or by looking at the tester repeatedly for help. When evaluating the video, calculate the time between the disappearance of the rug and the time the pup stops searching. Register this time.

Test element 10: food

The tester takes a small pellet of dry food (usual puppy food) and offers it to the pup.

Evaluation 10: does the pup take the food?

- -: the pup does not take the food
- +: the pup takes the food

Test element 11: passive plastic bag

The tester takes a plastic bag, stretches it out between his hands, puts it down on the floor in front of the chair and sits down.

Evaluation 11: the degree of ease of the pup with to this creaky plastic bag is observed.

- --: pup is afraid, or does not approach the plastic bag at all
- -: pup approaches very cautiously, slightly sniffing, or shows very little interest after first approach
- +: pup is interested, sniffs it, licks or nibbles it, moves on top of it
- ++: pup is highly interested and either picks it up immediately and walks around with it, or immediately lies on top of it chewing it or guarding it

Test element 12 & 13: active bag and distraction

The tester takes the plastic bag and traces it over the ground in a figure 8 whilst seated, allowing the pup to chase and if he is quick enough to catch it. He repeats this 3 times with a short interval (10-20 sec). After the third time, the tester taps on the ground with a pen close to the bag (30 cm).

Evaluation 12: the manner in which the pup reacts to the moving bag is evaluated. If there is a significant change in which the pup follows the bag between the 3 different throws, this can be marked by evaluating the first chase with 1 and the final chase with 2 in the relevant boxes of the form.

- --: pup is not interested or afraid and does not chase the bag at all
- -: pup is interested and follows the movement of the bag only after a lot of movement OR lets go of it quickly after it is still
- +: pup is interested and follows the rug but does not really try to catch it and not at a high speed

- ++: pup follows in a high speed chase, really trying to catch it, or grabbing it

Evaluation 12A: After a + or a ++ in 12; does the pup show signs of retrieving it?

- -: the pup smells at the bag but does not pick it up in his mouth
- +: the pup takes the bag in his mouth and moves around near the tester
- ++: the pup bites the bag intensely, he may shake it, and then moves towards the tester

Evaluation 12B: After a + or a ++ is 12; does the pup show prey-related behaviour? If so, evaluate (score the weakest behaviour!):

- --: pup defends the bag when the tester tries to take it away by biting the tester immediately
- - pup defends the bag when the tester tries to take it away by growling and a stiff body posture
- +: pup stays on rug and nibbles it, or picks it up and actively evades tester, or shows demonstration behaviour showing off prey
- ++: pup stays on rug, tucking it in, and starts chewing it or takes it fully into its mouth

Evaluation 13: the manner in which the pup responds to the distraction is noted

- --: the pup growls and defends the bag, tail immobile, stiffened body posture
- -: pup is easily distracted and happily changes its attention to the pen,, or has a slightly defensive body posture and maybe attacks the hand
- +: pup is not easily distracted at all but stays alert with waving tail but does not respond to the pen
- ++: pup moves quickly towards the pen ("can't help himself"), but returns to the bag in a flash

Test element 14: disappearance of plastic bag

The tester stands up, and makes a 4th figure of 8 on the ground in front of him, preventing the pup from catching the bag. Then with a quick motion he pulls the bag up from the floor and hides it under his arm, and remains standing for at least 30 seconds, or until the dog has stopped searching.

Evaluation 14: the manner in which the pup reacts to the sudden disappearance of the bag is evaluated.

- --: the pup does not search for the bag at all
- -: the pup looks around for <20 seconds or comes back to the tester for "help"
- +: the pup continues looking for it for up to 40 seconds, OR only visually, OR not really intensely
- ++: the pup continues to look for it intensely AND for longer than 40 seconds AND using its nose

Evaluation 14A: if the pup scores a ++ in 5, wait until the pup stops searching, either by changing activity or by looking at the tester repeatedly for help. When evaluating the video, calculate the time between the disappearance of the bag and the time the pup stops searching. Register this time.

Test element 15: metal chain

The tester hides a metal chain (eg collar) in his hand and sits down. He throws the chain on the floor whilst the pup is standing with his head away from him so the pup does not see where the chain comes from, and throws it so it lands approximately 1-1.5m away to the side of the pup.

Evaluation 15: the reaction of the pup to (the sound of) the falling chain is observed.

- --: afraid of the sound, stays far away from the chain
- -: afraid of the sound but recovers slightly, does not approach the chain fully
- +: reacts but recovers, approaches the chain and smells it, maybe nibbles
- ++: not afraid at all, direct interest and approach

Evaluation 15A: After a + or a ++, what does the pup do next?

- -: pup does not pick up the chain
- +: pup picks up the chain
- ++: pup picks up the chain, runs around with it

Test element 16: on back

The tester sits down on the floor and allows the pup to come to him, gently picks him up, and puts the pup on his lap on its back, holding the pup so that it stays on its back in his lap while gently stroking its belly. He holds the pup like this appr. 30 sec.

Evaluation 16: the behaviour of the pup in this position is observed, and the tester adds his own evaluation of the degree of tension he feels in the pup in the remarks.

- --: pup shows extreme fear, or continuously struggles with its tail between its legs
- -: pup continues to struggle but the tail is relaxed
- +: the pup struggles a little but is relaxed for the major part of the time
- ++: the pup is completely relaxed all of the time

Appendix B

Laboratory protocols

DNA isolation - Blood

DNA was isolated from blood samples using the E.Z.N.A® Blood DNA Mini Kit Protocol. Buffers were prepared according to directions in the instruction manual. Elution buffer were heated to 65°C.

1. 250 µL of blood were transferred to a sterile micro centrifuge tube.
2. 25 µL OB Protease and 250µL were added to sample. Vortexed at maximum speed for 15 seconds.
3. Sample was incubated at 65°C for 10 minutes and briefly vortexed (1-2s) during incubation (4-6 minutes into incubation)
4. 260 µL 100% Ethanol were added. Vortexed tubes at maximum speed for 20 seconds.
5. Sample were briefly centrifuged to collect any drops from inside the lid.
6. Inserted a HiBind® DNA Mini Column into a 2 mL collection tube
7. Transferred the entire sample to column.
8. Centrifuged the sample at ≥10000 RPM for 60 seconds.
9. Discarded the filtrate and kept the collection tube.
10. HiBind® DNA Mini column was inserted into a new 2 mL collection tube
11. 500 µL HBC buffer was added
12. Sample was centrifuged at ≥10000 RPM for 60 seconds.
13. Filtrate was discarded and collection tube was reused.
14. 700µL was buffer was added.
15. Sample was centrifuged at ≥10000 RPM for 60 seconds.
16. Filtrate was discarded and collection tube were reused.
17. Steps 14-16 were repeated.
18. The empty HiBind column were then centrifuged at 13.000 RPM for 2 minutes.
19. The column was then transferred to a 2 mL microcentrifuge tube.
20. 150 µL of Elution Buffer heated to 65°C was added.
21. Sample was then incubated at room temperature for 5 minutes.
22. The Sample was centrifuged at 13.000 RPM for 60 seconds.
23. The filtrate was reused and added to the column again to increase yield, without sacrificing concentration. Centrifuged at 13.000 RPM.
24. DNA was stored at -20°C

DNA isolation – buccal swab

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

1. The sample was manually shaken for a few seconds.
2. The sample was incubated in the thermomixer at 50 ˚C for a minimum of one hour.
3. The cap was removed and the collection sponge was pressed against the inside of the tube to extract as much of the sample as possible.
4. The sponge was cut of the cap with scissors and discarded.
5. 500 µL of the mixed Performagene sample was transferred to an Eppendorf tube. The cap was put back on the original tube and the rest of the sample was stored at -20 degrees Celsius.
6. 20 µL of PG-L2P purifier was added to the sample and the sample was vortexed for a few seconds.
7. The sample was incubated on ice for 10 minutes.
8. The sample was centrifuged for 5 minutes at 15000 x g.
9. The supernatant was carefully transferred to a fresh Eppendorf tube. The pellet and old tube was discarded.
10. 25 µL of 5 M NaCl solution was added to the sample.
11. 600 µL of room temperature 100% ethanol was added to the sample.

12. The sample was gently mixed by inversion 10 times.
13. The sample was then left at room temperature for 10 minutes.
14. The tube was placed in the centrifuge in a known orientation and centrifuged for 2 minutes at 15000 x g.
15. The supernatant was carefully removed with a pipette, without disturbing the DNA pellet.
16. The DNA was washed by adding 250 µL of 70% ethanol.
17. The ethanol was carefully removed after 1 minute.
18. The tube was centrifuged briefly to pool any remaining ethanol so that it could be removed.
19. The DNA pellet was resuspendated in 100 µL TE buffer. Then the sample was vortexed.
20. The sample was left at room temperature overnight (or for a couple of days), and vortexed briefly a couple of times during the incubation.

Appendix C

Table 5: Primers used for investigating variation in HTR2A gene.

Primer name	Sequence	Description
HTR2A_MS2_F	GGACTCGATCCCAGGTCTC	Microsatelite
HTR2A_MS2_R	TCAGCAGGGAATCTGCTTCT	
HTR2A_MS2_R new	CATGACCTCAGGGTTGTGAG	New reverse primer
HTR2A-S2-3_F	GACCTCCTCGTTTGCCACT	SNP found in literature (van den Berg et al., 2005)
HTR2A-S2-3_R	CTTCCATGACCTGGGCTTT	
HTR2A-S1_F	GCTTTGGGACAAGGACACTG	SNP found in literature (van den Berg et al., 2005)
HTR2A-S1_R	GGGAGTTGATGGGAGATGGT	
HTR2A-usb_F	ACGCCTGGAACCACAAAACT	Upstream region, part b
HTR2A-usb_R	CGTTTGACGGCATTAAGGAG	
HTR2A-usa_F	CGACTGCTGCTCTCCTCCT	upstream region, part a
HTR2A-usa_R	TCCTTCAAAGCAAGGTCAAAA	
HTR2A-e3c_F	ACCTACAGGTCGGCCTTCTC	Exon 3 part c
HTR2A-e3c_R	TTGTTGGTTCTACTAGACTGGCTTT	
HTR2A-e3b_F	TCTTTCAGCTTCCTCCCTCA	Exon 3 part b
HTR2A-e3b_R	TGTGCTCTTGGCATCTTTCTT	
HTR2A-e3a_F	CCACGTCGAAATAGAATCCAG	Exon 3 part a
HTR2A-e3a_R	AAGAAGACAATGCCCAGCAC	
HTR2A-e2_F	CTCCATGGAAACCCTCCTG	Exon 2
HTR2A-e2_R	CCATAGTCACCGTGTCAGGTT	
HTR2A-e1a_F	GCTTCCGTGTGACAGAGACA	Exon 1 part a
HTR2A-e1a_R	TGATGACCAGGATGTTTCCA	
HTR2A-e1b_F	TAGCTGGTCAGTGGATGCAG	Exon 1 part b
HTR2A-e1b_R	AATAAACCCTGGTGGTCAGC	
HTR2A-MS1_F	GGATCAGCTCTCCAACCAGT	CA17 microsatellite ch22:4.459.820
HTR2A-MS1_R	AAATCACAATGCTCCCCAAG	
HTR2A-MS1_Rny	TTACTGCTGGTTGCACCTTG	New reverse primer
HTR2AmsTAGA11F	ATCCCAATTCCAGGCTCATA	Microsatellite
HTR2AmsTAGA11R	CAGGTGCCCCTTAGATACACA	
HTR2A-MS1F	GGATCAGCTCTCCAACCAGT	Microsatellite
HTR2A-MS1_R	TTACTGCTGGTTGCACCTTG	New reverse primer
HTR2AmsCA17F FAM-	TGCACCGCAATGTTTATAGC	CA17 microsatellite 22:4.453.724
HTR2AmsCA17R	TTCAATCCGTGTTGTTGCAT	
HTR2AmsCA18F FAM-	TCCCCTTTACAATTGCACCT	Microsatellite
HTR2AmsCA18R	TCCATGAGCATGGGATATCTT	
HTR2AmsAAAT9F	GGATCGAGTCCCACATCG	Microsatellite
HTR2AmsAAAT9R	AGAGGGAAAGGGGAGAACCT	

Appendix D

PCR and sequencing

The following protocol for PCR was used (Table D1, Table D2)

Table D1 – PCR solution	Sequencing (µL)	Fragment analysis (µL)
dH_2O	10,35	11,5
10x PCR buffer	1,5	1,5
dNTP mix (2,5 mM)	0,5	0,5
Forward primer	0,5	0,25
Reverse primer	0,5	0,25
Taq DNA polymerase (5U/µL)	0,05	0,05
q-solution	0,3	0
DNA sample	1,5	1,0

Table D2 – Protocol for PCR

Stage	Temperature	Duration	Cycles
1	95°C	2 minutes	1x
2a	96°C	30 seconds	29x
2b	59°C	40 seconds	
2c	72°C	50 seconds	
3	4°C	Until removed.	1x

Gel electrophoresis

All PCR product were tested using gel electrophoresis on a minimum of 10 samples

The products were run through a 0.5 cm thick gel consisting of a 1% agarose 1xTAE buffer mix, and a droplet Etidium bromide.

The current used was 180 volts for 20-30 minutes.

123bp or 100 bp ladder was used as positive control.

Primers without satisfactory yield were retested on a PCR-testing protocol, with stage 2b either lowered to 55°C or on a gradient of temperatures from 55°C to 60°C.

Sequencing

A BigDye kit was used for sequencing of PCR-products. For each sequence reaction, the protocol summarized in table D3 was used (Table D3)

Table D3 – Sequencing PCR	One sequence reaction (µL)
dH_2O	5,2
Bigdye Buffer	2
F or R primer	0,32
Big Dye terminator mix	1,5
PCR product	1

The solution was then exposed to the sequence summarized in Table D4

Table D4 – Sequencing PCR protocol

95°C	1 minute	1x
96°C 50 °C 60 °C	15 seconds 10 seconds 2 minutes	29x
4 °C	Until removed.	1x

Fragment analysis

24µL of a 500LIZ size standard (AB) were mixed with 1000µl of Furosemide, and then 9µL of this mix, was mixed with 1µL of each PCR product.

Results were analysed using Genemapper 5.1. (AB). Products with peaks lower than 200Hz were disregarded as they could not be reliably differed from background noise.

Alleles were named after their fragment lengths and corresponding loci e.g. “HTR2A MS1 290” allele is the 290 base pair long fragment of the MS1 locus (ch22:4.459.820) of the HTR2A gene.

Appendix E

The following table is copied "as is" from GTC, however the thresholds for Phenotype column is new for this study.

Broad phenotype	Traits	Thresholds for phenotype
Shyness/ boldness	**Test element 1 & 2: room and tester** The tester sits still with hands crossed over his chest for about 1.5 min, not seeking contact with the pup. During this time the behaviour of the dog is observed with regard to its investigation of the room (1), and with regard to the tester (2). *Evaluation 1*: The more free and easy the dog is in the room, the better. 1. --: freezing, sitting still and whining (if this happens the test is terminated) 2. -: cautiously walking around, front legs first so that the pup becomes really long, ("long Malinois") barking at things and people (if this happens the test may also be terminated, or the tester may interact a little with the pup as a "warm up") 3. +: walking around looking at things 4. ++: really investigating the whole room, using its nose *Evaluation 2*: Relaxed, friendly contact is the best. 1. --: no contact at all with the tester is not at all desirable 2. *[excluded for not being in a linear relationship with the other marks]* 3. +: contacting the tester very cautiously at first ("sneaky contact") and then coming a little closer 4. ++: friendly contact, occasionally jumping up against the tester is fine	Bold individuals were defined as having a sum of Test element 1+2 ≥ 7 Shy individuals were defined both as sum ≤5, to more clearly separate the phenotypes, and at ≤6 to increase power and investigate whether there still was a difference in ratio.
Playfulness	**Test 4: Active ball**. The tester picks up the ball and rolls it gently to one corner of the room. Depending on the behaviour of the pup, he takes it back from the pup or he goes and gets it. He repeats this a second t ime towards the same corner, and later a third time in another direction of the room. *Evaluation 4*: the manner in which the pup reacts to the moving ball is evaluated. If there is a significant change in which the pup follows the ball between the 3 different throws, this can be marked by evaluating the first roll with 1 and the final roll with 2 in the relevant boxes on the form. 1. --: the pup just looks at the ball rolling away and does not chase at all, maybe shows fear. 2. -: the pup follows the ball a short way, not to the end. 3. +: the pup follows the ball all the way to the end slowly 4. ++: the pup chases the ball at high speed. **Test element 6: passive rug** The tester takes a leather rug [Familiar toy] and stretches it between his hands, puts in on the floor in front of him and sits down. *Evaluation 6*: degree of ease with this familiar object is observed. 1. --: pup is afraid, or does not approach the rug at all 2. -: pup approaches very cautiously, slightly sniffing, or shows very little interest after first approach 3. +: pup is interested, sniffs it, licks or nibbles it, moves on top of it 4. ++: pup is highly interested and either picks it up immediately and walks around with it, or immediately lies on top of it chewing it or guarding it **Test element 7 active rug** the tester takes the leather rug and traces it over the ground in a figure 8 whilst seated, allowing the pup to chase and if he is quick enough to catch it. He repeats this 3 times with a short interval (10-20 sec). After the third time, the tester taps with a pen on the ground about 30cm away from the rug. *Evaluation 7*: the manner in which the pup reacts to the moving rug is evaluated. If there is a significant change in which the pup follows the rug between the 3 different figures of 8, this can be marked by evaluating the first chase with 1 and the final chase with 2 in the relevant boxes of the form.	To correct for the lack of completion of all the playfulness observations and average was used instead of the sum 5 dogs did not complete any of the playfulness tests and were not included. Playful was defined as an average score ≥3,75 And non-playful were defined as an average score ≤3,25, excluding the individuals scoring between 3,25 and 3,75 (n=22) to more clearly separate the phenotypes

	1. --: pup is not interested or afraid and does not chase the rug at all 2. -: pup is interested and follows the movement of the rug only after a lot of movement, OR lets go of it quickly after having it is still 3. +: pup is interested and follows the rug but does not really try to catch it and not at a high speed 4. ++: pup follows in a high speed chase, really trying to catch it, or grabbing it **Test element 12 & 13: active bag and distraction** The tester takes the plastic bag and traces it over the ground in a figure 8 whilst seated, allowing the pup to chase and if he is quick enough to catch it. He repeats this 3 times with a short interval (10-20 sec). After the third time, the tester taps on the ground with a pen close to the bag (30 cm). *Evaluation 12*: the manner in which the pup reacts to the moving bag is evaluated. If there is a significant change in which the pup follows the bag between the 3 different throws, this can be marked by evaluating the first chase with 1 and the final chase with 2 in the relevant boxes of the form. 1. --: pup is not interested or afraid and does not chase the bag at all 2. -: pup is interested and follows the movement of the bag only after a lot of movement OR lets go of it quickly after it is still 3. +: pup is interested and follows the rug but does not really try to catch it and not at a high speed 4. ++: pup follows in a high speed chase, really trying to catch it, or grabbing it	
High search intensity	**Test element 5: disappearance ball** The tester takes the ball in his hand and makes three figures of 8 with it on the floor. The pup is usually interested in this and will follow the hand with the ball. Then the tester quickly moves his hand with the ball away from the floor behind his back, he remains seated for at least 30 seconds, or until the pup has stopped searching. *Evaluation 5*: the manner in which the pup reacts to the sudden disappearance of the ball is evaluated. 1. --: the pup does not search for the ball at all 2. -: the pup looks around for <20 seconds or comes back to the tester for "help" 3. +: the pup continues looking for it for up to 40 seconds, OR only visually, OR not really intensely 4. ++: the pup continues to look for it intensely AND for longer than 40 seconds AND using its nose **Test element 9: disappearance rug** The tester stands up, and makes a 4th figure of 8 on the ground in front of him, preventing the pup from catching the rug. Then with a quick motion he pulls the rug up from the floor and hides it under his arm, and remains standing for at least 30 seconds, or until the pup has stopped searching. *Evaluation 9*: the manner in which the pup reacts to the sudden disappearance of the rug is evaluated. 1. --: the pup does not search for the rug at all 2. -: the pup looks around for <20 seconds or comes back to the tester for "help" 3. +: the pup continues looking for it for up to 40 seconds, OR only visually, OR not really intensely 4. ++: the pup continues to look for it intensely AND for longer than 40 seconds AND using its nose *Evaluation 9A*: if the pup scores **Test element 14: disappearance of plastic bag** The tester stands up, and makes a 4th figure of 8 on the ground in front of him, preventing the pup from catching the bag. Then with a quick motion he pulls	Case/Control design A High Search intensity was defined as an average of 4, and non-searching was defined as average of ≤3,33 excluding 20 dogs with scores between 3,33 and 4 Case/control design B To increase power we also defined a non-searching group to be every dog with an average of <4.

	the bag up from the floor and hides it under his arm, and remains standing for at least 30 seconds, or until the dog has stopped searching. *Evaluation 14*: the manner in which the pup reacts to the sudden disappearance of the bag is evaluated. 1. --: the pup does not search for the bag at all 2. -: the pup looks around for <20 seconds or comes back to the tester for "help" 3. +: the pup continues looking for it for up to 40 seconds, OR only visually, OR not really intensely 4. ++: the pup continues to look for it intensely AND for longer than 40 seconds AND using its nose	
Fear of humans	**Test element** *2* 1. --: no contact at all with the tester is not at all desirable 2. [excluded for not being part of a linear relationship with the other marks] 3. +: contacting the tester very cautiously at first ("sneaky contact") and then coming a little closer 4. ++: friendly contact, occasionally jumping up against the tester is fine	Fearful phenotype was defined as having a score of 1 and scoring at least 2 or higher on Evaluation 1 to reduce the risk of confounding influence from generalized anxiety A second definition of fearful was having ≤3 on this test. This was done to increase power. Non-fearful phenotype was defined as scoring 4 on this test.
Startle reflex	**Test element 15: metal chain** The tester hides a metal chain (eg collar) in his hand and sits down. He throws the chain on the floor whilst the pup is standing with his head away from him so the pup does not see where the chain comes from, and throws it so it lands approximately 1-1.5m away to the side of the pup. *Evaluation 15*: the reaction of the pup to (the sound of) the falling chain is observed. --: afraid of the sound, stays far away from the chain -: afraid of the sound but recovers slightly, does not approach the chain fully +: reacts but recovers, approaches the chain and smells it, maybe nibbles ++: not afraid at all, direct interest and approach	No startle reflex phenotype was defined as scoring 4 on this test. Positive startle reflex phenotype was defined as scoring 3 or lower on this test.
Interact chain	**Test element 15: metal chain** The tester hides a metal chain (eg collar) in his hand and sits down. He throws the chain on the floor whilst the pup is standing with his head away from him so the pup does not see where the chain comes from, and throws it so it lands approximately 1-1.5m away to the side of the pup. *Evaluation 15A*: After a + or a ++ [in Evaluation 15], what does the pup do next? -: pup does not pick up the chain +: pup picks up the chain ++: pup picks up the chain, runs around with it	No interaction phenotype was defined as scoring 1 on this test. High interaction was defined as scoring 3 on this test. Individuals scoring 2 were excluded to achieve a separation of phenotypes
Cautious/curious	**Test element 3**: **passive ball** The tester puts a tennis ball in front of him on the floor, taking care that it does not roll away. He times the placing of this ball in such a way that the pups attention is directed towards this ball.	Cautious phenotype defined as scoring 1 or 2

	Evaluation 3: Degree of ease with this new object is evaluated. --: the pup is afraid of this ball and does not approach it -: pup approaches the ball very cautiously, slightly sniffing, or shows very little interest at all + direct and continued interest, but just sniffing it and staying with it, maybe followed by taking it in its mouth after a while ++ direct interest followed by taking it in its mouth immediately	Curious phenotype defined as scoring 4. Individuals scoring 3 were excluded to achieve a separation of phenotypes