# Genetics of aggressive behaviour in Golden Retriever dogs

Genetica van agressief gedrag bij de Golden Retriever
(met een samenvatting in het Nederlands)

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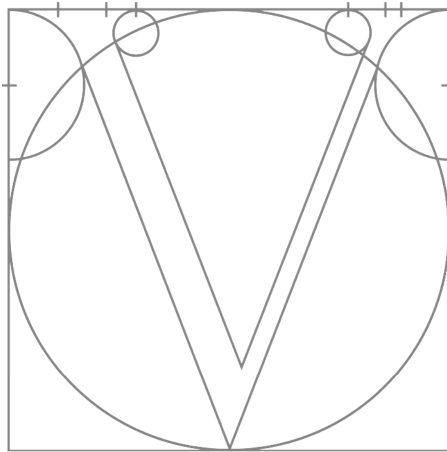

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**Cover:** The illustration on the cover depicts a fundamental question about canine aggression: Are aggressive dogs wolves in sheep's clothing or are they sheep in wolves' clothing? (Illustrations by Linda van den Berg)

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# CHAPTER 1

# General introduction

# 1.1
# Background and outline of the thesis

Understanding the relation between genes and behaviour is one of the most challenging goals in neurobiology. The research described in this thesis aimed to identify genetic variations underlying the variation in aggressive behaviour in Golden Retriever dogs.

*A Golden Retriever is by nature gentle, friendly and trustworthy at all times. He is also courageous, intelligent, easy to train, and has a great desire to please his owner. It is a combination of these attributes which makes him the ideal family companion or shooting dog. A Golden which shows any sign of having too highly developed a guarding instinct, any tendency to bite, aggressiveness or nervousness, has not the true Golden temperament. These differing faulty temperaments are hereditary and dogs possessing them should never be bred from. Admittedly, such failings can be intensified by a bad environment and incorrect treatment, but I maintain that there must be some inherent tendency towards them in the dog for them to be displayed.*

Tudor 1980

In spite of their friendly reputation, Golden Retrievers were the most frequently encountered purebred dogs in a group of Dutch dogs diagnosed with fear-motivated aggression (Galac and Knol 1997). Although this may in part reflect the high number of Golden Retrievers in the Netherlands (Schellart and den Hertog 1998), it is surprising to encounter many aggressive individuals in a breed that has been selected for friendliness (Tudor 1980; http://www.goldenretrieverclub.nl/; link accessed August 2005).

Knol and Schilder (1998a) listed characteristics of aggressive Golden Retrievers based on descriptions provided by dog owners. In the majority of the Goldens the provocation was approaching or touching the dog. Threatening by the dogs was reduced to a minimum: the dogs growled while biting instead of preceding biting. The dogs displayed a low body posture with ears and tail down. Some dogs simultaneously expressed signs of dominance. Some dogs were exclusively aggressive towards humans, while others also attacked conspecifics. Although the owners considered this behaviour to be threatening, most of them were very attached to their dog and described the dog as friendly.

In a pilot study, Knol and colleagues (1997) analysed the genealogy of 30 aggressive Golden Retrievers and 66 randomly chosen dogs of the same breed. The total group of 96 dogs appeared to consist of two familial sub-

groups. The number of aggressive individuals differed significantly between these two subgroups (p=0.035). This was a first indication that genetic factors are important in the aetiology of aggression in Golden Retrievers.

Our studies of the genetics of aggression in Goldens started in 1997. In section 1.2 we argue why it is important to study aggression in dogs. Section 1.3 contains a review of the literature on the genetics of canine aggression. From 1997 to 2005, dogs were recruited by approaching owners of aggressive Golden Retrievers through advertisements in magazines for veterinarians, Golden Retriever breeders, or dog owners (Knol and Schilder 1998a; 1998b; 1999; and 2000; van den Berg *et al.* 2002; 2003; 2004a; 2004b; 2005; http://home.hccnet.nl/b.vanoost/). We collected behavioural information and DNA samples of 139 aggressive Golden Retrievers ("probands'). We asked owners of relatives of these probands to participate in our study as well. In 2005 we had behavioural information and a DNA sample of 138 relatives of 36 of the probands. A selection of the Golden Retriever families is depicted in the Appendix of section 3.4 (page 175-178).

Correct phenotyping is the key to success of genetic studies (Bearden *et al.* 2004; Mills 2003; Smoller *et al.* 1998). This is the theme of chapter 2 of this thesis. We initially phenotyped the dogs with a behavioural (aggression) test and a personal interview with the dog owner. In section 2.1, we describe the analysis of aggression tests of 83 Golden Retrievers. We expected the aggression test to provide objective and detailed behavioural phenotypes. However, it turned out to be difficult to elicit aggressive behaviour in the test. We therefore designed a mail questionnaire based on the successful canine behavioural assessment and research questionnaire (Hsu and Serpell 2003). All dog owners that had participated in our project earlier were asked to fill out the questionnaire. The results of this survey are described in section 2.2. In section 2.3 we describe a quantitative genetic assessment of the questionnaire scores. The aim of this work was to examine the usefulness of the behavioural measures for genetic studies. Based on the results, we made a selection of dogs for the molecular genetic studies presented in chapter 3.

We used a candidate gene approach in our search for molecular genetic variants that contribute to aggression in Golden Retrievers. Four genes of the canine serotonergic system were cloned, characterised, and studied in Golden Retrievers (sections 3.1, 3.2, and 3.3). We studied the serotonin receptor genes 1A, 1B, and 2A (*htr1A, htr1B, htr2A*) and the serotonin transporter gene (*slc6A4*). In section 3.4, we describe the analysis of these genes in related Golden Retrievers. We conclude that the candidate genes are not likely to play a major role in the variation in aggression in the Golden Retrievers. The thesis ends with a general discussion of the results (chapter 4).

# 1.2
# Why study canine aggression?

The influence of heredity on behaviour has been debated for centuries (Plomin 1990). Although it is generally agreed that behaviour is affected by genes, a lot of controversy remains (Hamer 2002; Robinson 2004). Evidence for an association between specific genes and behavioural traits is often inconclusive (Bearden *et al.* 2004; Colhoun *et al.* 2003; Hamer 2002; Inoue and Lupski 2003).

Dog breeds are very suitable for exploring the genetics of behaviour. A dog can only be registered as a member of a breed if both its sire and dam are registered members of the same breed. As a result, dog breeds are genetic isolates. The low genetic heterogeneity within dog breeds implies that only a limited number of the genes that influence a certain behavioural trait will be functionally polymorphic within a breed. This greatly facilitates the chances of finding such genes. The uniformity within breeds is contrasted by the extraordinary variation in morphology and behaviour between breeds. This combination of intrabreed homogeneity and interbreed diversity provides unparalleled opportunities for elucidating the genetics of behaviour. Therefore, studying the genetics of canine aggression can add in a fundamental way to the nature and nurture debate.

In addition to its fundamental scientific importance, studying canine aggression has an obvious applied function. In the Netherlands, each year about 240 people are hospitalised as a consequence of dog bites (Mulder 1991; Schellart and den Hertog 1998; Toet and den Hertog 2000). Aggression is the most common problem in dogs encountered by behavioural specialists (Borchelt 1983; Landsberg 2004; Mugford 1984). Treatment of aggression problems usually involves changing environmental factors through behavioural therapy and sometimes medication is added (e.g. Landsberg 2004; O'Farrell 1991; Overall 1997; Reisner 2003; Voith 1984). However, this has limited success and aggression is therefore a common reason for euthanatizing dogs (Mikkelsen and Lund 2000). Understanding the genetic factors underlying aggression leads directly to the relevant pathophysiological mechanisms of aggression. This can guide pharmacological intervention for which scientific evidence is sparse to date. In addition, aggression problems could be reduced through breeding programs.

A third reason for studying aggression in dogs is the usefulness of canine behavioural problems as a model for human mental disorders (Overall 2000). Both humans and dogs descend from social species that show coope-

ration and mutually helpful behaviour. As pointed out above, behavioural traits can be more easily mapped in canine than in human populations. Once we have identified causal mutations in canine genes, it will be interesting to study polymorphisms of the corresponding genes in humans.

Finally, dogs fulfil important functions in our society as working dogs, e.g. police dogs and guide dogs for the blind. Most guide dog failures are caused by behaviour problems (Goddard and Beilharz 1982; 1983; Serpell and Hsu 2001). Breeding programs for working dogs would benefit substantially from insight into the genetics of canine behaviour.

two Golden Retrievers

# 1.3
# Review of the genetics of canine aggression

In this section we review literature related to the genetics of canine aggression. We will start at the level of behaviour and then zoom in to the level of the single gene. This review is not intended to be exhaustive, but is aimed at providing a framework for understanding the research described in this thesis.

## Phenotypes of aggression

*The chief difficulty is to define the condition the heredity of which one is attempting to trace.*
Felix Brown 1942

Dogs are wolves in sheep's clothing. The grey wolf is considered to be the progenitor of the domestic dog (Vila *et al.* 1997). Aggression influences the chances of survival and reproductive success in wolves. Wolves use aggression to defend themselves from predators and to compete for food, social status, or reproduction. Situations that elicit aggressive behaviour in wolves can also elicit aggression in dogs. Scott and Fuller (1965) observed that the basic patterns of agonistic behaviour are similar in dogs and wolves. Agonistic behaviour includes behavioural elements such as retreat, submission, and flight in addition to aggressive behavioural elements. However, selection has modified the frequency of expression of agonistic behaviour. As a result, dogs and wolves display similar patterns of fighting when sufficiently aroused, but there is a difference in the amount of stimulation required to trigger this reaction (Scott and Fuller 1965). Although canine aggression is usually normal behaviour (Borchelt and Voith 1996; Mills 2003; Mugford 1984; Reisner 1997), it can lead to unacceptably dangerous situations (Netto and Planta 1997; Winkler 1977; Wright 1985). Some authors consider aggression to be a disorder when the behaviour is too intense, too prolonged, too frequent, or when it occurs in the absence of an appropriate stimulus (Jacobs 2003; Mills 2003; Overall 1997).

It appears to be impossible to formulate a simple, precise, and unitary definition of aggression (Berman and Coccaro 1998; Borchelt 1983; Borchelt and Voith 1996; Eichelman 1987; Ferris and de Vries 1997; Moyer 1976; Peremans 2003; Ursin and Olff 1995; Valzelli 1981; Vitiello and Stoff 1997; Wiepkema and van Hooff 1977). In this thesis, we define canine aggressive

behaviour in terms of the aggressive behavioural elements, e.g. growling, baring the teeth, and biting. These behavioural elements can be part of various behaviour systems, including agonistic behaviour, predatory behaviour, and play behaviour. Most cases of problematic aggression in dogs reported to vets involve agonistic behaviour.

## Aggression subtypes

There are several kinds of aggression and there are indications that the subtypes have a distinct genetic basis (Popova *et al.* 1993). This suggests that aggressive phenotypes should be split into specific classes for genetic studies. Unfortunately, it is not agreed upon how aggression should be subdivided (Houpt and Willis 2001; Lesch and Merschdorf 2000; Serpell and Jagoe 1995). One of the first classifications of animal aggression was based on the type of situation evoking the behaviour, with the following classes: predatory, inter-male, fear-induced, irritable, territorial, maternal, instrumental and sex-related aggression (Moyer 1976; Table 1). Predatory aggression is stimulated by a natural object of prey. Inter-male aggression is evoked by the presence of an unknown male conspecific. Fear-induced aggression is stimulated by an inescapable threat. A broad range of stimuli can elicit irritable aggression. Territorial aggression is elicited by the presence of an intruder in the home territory of the resident. Maternal aggression is evoked by a threat to the young of a female. Instrumental aggression is a learned response that occurs because it has been reinforced in the past. Sex-related aggression is stimulated by the same stimuli which produce sexual response.

Moyer (1976) suggested that each class has a distinct neural and endocrine basis. For instance, it was found that stimulation of an area in the lateral portion of the basal nucleus of the amygdala of cats facilitates fear-induced aggression, but inhibits both predatory and irritable aggression. More recent studies have suggested a distinct genetic basis of Moyers aggression classes. Selection of rats and silver foxes for reduced fear-induced aggression towards humans did not change predatory or inter-male aggression (Naumenko *et al.* 1989; Popova *et al.* 1993). However, reduced aggressiveness towards man in the rats and foxes was accompanied by reduced fear of novelties and irritable aggression, indicating that there is some overlap between the classes. Moyer also noted that his classes were not mutually exclusive.

Many researchers have used modified versions of Moyers classification (Valzelli 1981; Vitiello and Stoff 1997). Some have re-grouped the aggression subtypes in two general classes: defensive and offensive aggression (Blanchard and Blanchard 1984; Borchelt 1983; Moyer 1976; Reis 1971; Siegel *et al.* 1999;

**Table 1.** Examples of functional classifications of canine aggression. The classification of Moyer (1976) has been included (left end of the table).

| Moyer 1976 | Beaver 1983 | Hart and Hart 1985 | O'Farrell 1991 | Borchelt and Voith 1996 | Overall 1997 | Houpt 1998 | Reisner 2003 | Landsberg 2004 |
|---|---|---|---|---|---|---|---|---|
| - | competitive (dominance)[1] | dominance-related | dominance | dominance | dominance | social or dominance-related | owner-directed | dominance-related |
| - | - | - | - | - | - | - | - | conflict-related |
| - | - | competitive | - | possessive | food-related/ possessive | - | food-related | possessive/ food-related |
| fear-induced | fear-induced | fear-related | - | fear | fear | fear-induced | fear-related | fear-related |
| territorial | protective | territorial | territorial/ protective[2] | protective | territorial/ protective | territorial | territorial | territorial/ protective |
| - | medically related | - | - | pathophysio logical | - | - | - | pathophysio logical |
| predatory | predatory | predatory | predatory | predatory | predatory | predatory | predatory | predatory |
| irritable | pain-induced | pain-induced | pain-induced [2] | pain-elicited | pain | pain-induced | - | pain/medical/ irritable |
| - | playful | | - | - | play | - | - | play |
| maternal | maternal | maternal | maternal [2] | maternal | maternal | maternal | - | maternal/hormonal |
| - | redirected | - | - | redirected | redirected | - | - | redirected |
| instrumental | learned | learned | - | - | - | - | - | learned |
| intermale | intermale | intermale | dominance towards other dogs[1] | intermale interfemale | interdog | - | - | intraspecific |
| - | - | - | - | punishment-elicited | - | - | - | - |
| - | - | idiopathic | - | - | idiopathic | - | - | idiopathic |
| sex-related | sexual | - | - | - | - | - | - | - |
| - | - | - | rage syndrome[2] | - | - | - | - | - |
| | | | | | | | | |

[1] Description of the aggression class differs from descriptions of other authors.

[2] Presented as a variant of dominance aggression.

Wright and Nesselrote 1987). Others have proposed the general classes of affective and non-affective aggression (Archer and Browne 1989; Beaver 1983; Dodge and Coie 1987; Eichelman 1981; Jacobson 1996; Malone *et al.* 1998; Scarpa and Raine 1997; Vitiello *et al.* 1990; Vitiello and Stoff 1997). Affective aggression involves an intense patterned autonomic response and includes irritable, inter-male, fear-induced, and maternal aggression (Eichelman 1981). Non-affective aggression is not accompanied by an autonomic response and includes predatory aggression. This subdivision is similar to the dichotomy of impulsive (reactive) aggression and controlled (proactive) aggression that is often used in human aggression research (Buss 1961; 1966; Feshbach 1964; Kempes *et al.* 2005; Vitiello and Stoff 1997).

Most classifications of canine aggression are modified versions of the classification proposed by Moyer (1976). They are usually referred to as "functional classifications" because one can make inferences about the function of the behaviour by considering the context in which it occurs. We have listed several functional classifications of canine aggression in Table 1. These classifications have been made for clinical purposes. Most classifications include more classes than the original classification of Moyer. The classes of canine aggression are not strictly separated. Many authors reported that dogs regularly exhibit more than one type of aggression (Beaver 1993; Landsberg 2004; Overall 1997). In addition, aggression problems were often associated with problems of anxiety (Borchelt 1983). Although the classifications may be practical in the clinic, the biological meaningfulness of the classes is unclear. The classes do not seem to reflect biological processes and are therefore not useful for genetic studies.

A few authors have proposed alternative classifications of canine aggression (reviewed by Borchelt and Voith 1996 and Jacobs 2003). An example is a classification based on the target of aggression, such as the owner, strangers, or animals (Borchelt and Voith 1996; Houpt 1998; Mugford 1984; Reisner 2003). Such classes may prove more useful, but they may also be too broad.

## Related behaviours and higher-order characteristics

Some researchers have suggested that aggression is part of a heritable higher-order trait such as coping style, tameness, or the personality trait of agreeableness (e.g. Benus *et al.* 1991; Draper 1995; Gosling *et al.* 2003; Gosling and John 1999; Trut 2001). In addition, many studies have reported correlations of aggression with other traits, such as anxiety and coat colour (e.g. Hemmer 1990; Houpt and Willis 2001; Podberscek and Serpell 1996; Trut

1999). Therefore, in some cases one may miss important information when using narrowly defined phenotypes, which also decreases statistical power.

An interesting example of a higher-order trait that includes aggressive behaviour is coping style in male wild house mice selected for short attack latency (SAL) and long attack latency (LAL) (van Oortmerssen and Bakker 1981). It has been suggested that the heritable differences in attack latency in these mice reflect two alternative strategies to cope with the environment (Benus *et al.* 1991). In addition to attacking intruders faster, the aggressive SAL mice display an active response to aversive situations. For instance, SAL mice show defensive burying of shock-delivering devices and they flee when they are confronted with a stronger male. The SAL mice are less flexible in their behaviour and consequently do not react to minor changes in their environment. Conversely, the non-aggressive LAL mice have a passive style of coping. They tend to react with immobility and withdrawal and show more flexible behaviour (Benus *et al.* 1991). The success of these alternative coping styles depends on the stability of the environment. The SAL phenotype performs better in a settled population, whereas the LAL phenotype is advantageous in migrating mice (Sluyter *et al.* 1996; van Oortmerssen and Busser 1989).

Aggression can also be viewed as the opposite of tameness. A long-lasting experiment at the Institute of Cytology and Genetics of the Siberian Department of the Russian Academy of Sciences in Novosibirsk has greatly improved our insight in the influence of selection for tameness on behaviour (see Trut 1999 or Trut 2001 for reviews). This research group uses silver foxes (*Vulpes vulpes*) as a model for dogs. Silver foxes are taxonomically close relatives of dogs. The experiment started in 1959 with 130 foxes from a commercial fur farm. The foxes were selected for tameness, resulting in foxes that are docile and eager to please (Belyaev 1979). After several generations of selection, the researchers observed dog-like physical characteristics in some of the foxes. For instance, some had a white spot on the head, loss of pigment in other areas, floppy ears, or rolled tails (Belyaev *et al.* 1981; see Figure 1). Their explanation for the observations was that behaviour and early development are both controlled by genes that regulate neural and endocrine systems.

A study of Svartberg and Forkman (2002) provides insight into the relationship between aggression and canine personality traits. Behavioural data from 1175 dogs of 47 breeds were investigated. These data were collected using the Swedish dog mentality assessment test (DMA). The DMA consists of ten subtests, during which dogs are exposed to several situations. Their reactions are recorded for 33 behavioural variables, including aggression-related measures. The investigators performed factor analysis on these

**Figure 1.** Loss of pigmentation in a silver fox selected for tameness at the Institute of Cytology and Genetics in Novosibirsk. The loss of pigmentation is determined by the homozygous state of the incompletely dominant autosomal *Star* mutation.

variables and extracted five primary factors: (1) playfulness, (2) curiosity/ fearlessness, (3) chase-proneness, (4) sociability, and (5) aggressiveness. Higher order factor analysis showed that all factors except aggressiveness were related to each other, creating a broad factor that influences behaviour in a range of situations. This broad factor is comparable to the shyness-boldness axis in humans and animals, which reflects a general tendency to approach novel objects and a willingness to take risks. This broad dimension is supported by earlier dog studies. Saetre *et al.* (2006) confirmed the existence of the shyness-boldness personality trait in the DMA data with an analysis of genetic variation. Their results suggest that there is shared genetics behind all behavioural traits except in those related to aggression. Scott and Fuller (1965) also noticed that timidity and aggressiveness were not opposite ends of the same continuum but were found in different factors. These results suggest that aggression forms a separate personality dimension. However, conclusions

about aggression based on behavioural tests should be treated with caution because it is not clear what type of aggression is measured with a test (see general discussion).

## Measuring aggression

Tools for measuring aggression include clinical diagnoses, questionnaires, behavioural tests, and behavioural observations. In genetic studies of human mental disorders, clinical diagnoses are often used as phenotypes (e.g. Ponce *et al.* 2003). The standard text which describes human mental illness is the *diagnostic and statistical manual of mental disorders* (DSM). The aim of the DSM is to develop a series of categorical diagnoses that are mutually exclusive and jointly exhaustive. The use of the DSM for phenotyping has been criticised because the diagnoses are not based on pathogenic mechanisms (Gottesman and Gould 2003; Mills 2003). This applies to clinical classes of canine aggression as well. Other researchers have used self-report questionnaires such as the Brown-Goodwin Aggression Scale (e.g. Baca-Garcia *et al.* 2004; Koller *et al.* 2003) and the Buss-Durkee Hostility Inventory (e.g. Coccaro *et al.* 1997) for phenotyping adult human aggression. Judgments by knowledgeable informants are often used to measure aggressiveness in children (e.g. Bartels *et al.* 2003; Matthys *et al.* 2001). Following this example, several researchers of canine behaviour have developed questionnaires for dog owners to evaluate the behaviour of dogs (e.g. Serpell and Hsu 2001; Hsu and Serpell 2003). A third method for measuring aggression is a behavioural test. Miczek *et al.* (2001) have reviewed methods of measuring aggression in mice. The "resident-intruder test" is the most frequently used. In this test, an isolated resident male is confronted with a group-housed strange mouse in the home cage of the resident or in a neutral cage. There are also many tests for dog behaviour (e.g. Netto and Planta 1997; Scott and Fuller 1965; Svartberg and Forkman 2002; van der Borg *et al.* 1991; Wilsson and Sundgren 1997). Finally, in animal research, observations of behaviour in more or less naturalistic environments have been applied for measuring aggression. The work of Scott and Fuller (1965) is a telling example of such studies in dogs.

In order to be useful for genetic studies, phenotypic scores should be reliable and valid. Jones and Gosling (2005) have reviewed studies of canine temperament. They found that the reliability and validity of the measures was promising in most studies. However, they also noted that there were few studies that actually reported reliability and validity.

### Conclusion

We can conclude from the above discussion that aggression appears in various forms and is related to various other traits. This has implications for phenotyping, with one of the main questions being "to lump or to split?"; that is: should we subdivide aggression into homogeneous classes (and if yes, how?) or should we lump various subclasses into categories (and add other traits). For molecular genetic studies it is important to work with well-defined phenotypes. This implies that splitting of types of behaviour is warranted. Once the responsible genes have been identified, the underlying common factors may become clear.

## Neurobiology of aggression: between genes and behaviour

### Anatomy of aggression

Emotions are regulated by several brain regions, including the amygdala, hippocampus, medial preoptic area, hypothalamus, anterior cingulated cortex, and ventral striatum (reviewed by Nelson and Chiavegatto 2001). The neural circuit regulating aggression seems to be rather conserved in mammals, but there are also differences between species. The prefrontal cortex has received much attention in studies of violent behaviour in humans (Enserink 2000; Goyer *et al.* 1994; Goyer and Semple 1996; Raine *et al.* 1994; Volkow and Tancredi 1987). The most important function of the prefrontal cortex, which is very large in humans, is to organise action in the time domain (Fuster 1997). Brain regions with sensory functions also play a role in the regulation of aggression. Moyer (1976) noted that all types of aggression were reduced by lesions in the lateral portion of the upper midbrain. This seemed to be due to the inability of the animal to receive and interpret sensory input. In a recent study, Mandiyan and colleagues (2005) showed that mice lacking a functional cyclic nucleotide-gated channel α2 do not mate or fight. This channel is required for signalling in the main olfactory epithelium in response to odours. Altered perception of stimuli can thus enhance or inhibit aggressive behaviour.

### Neurotransmitters and hormones

Aggression is mediated by intricate neural networks that use neurotransmitters as messengers. Several neurotransmitters have been reported to play a role in aggressive behaviour, including dopamine, γ-aminobutyric acid, and nor-

epinephrine (reviewed by Berman and Coccaro 1998; Brambilla *et al.* 2003; de Almeida *et al.* 2005; Miczek *et al.* 2002). Hormones influence the activity of neural networks. Some hormones have been suggested to be important mediators of aggression, most notably testosterone (reviewed by Giammanco *et al.* 2005) and the stress hormones that are released upon activation of the hypothalamic-pituitary-adrenocortical axis (Gulevich *et al.* 2004). There is evidence that these hormones affect canine aggression as well. For instance, male dogs are generally more aggressive than female dogs (Beaver 1983; Borchelt 1983; Hart and Hart 1985; Houpt 1998; O'Farrell 1991; Reisner 1997; Scott and Fuller 1965). This sex difference is likely to be mediated by androgens. Popova *et al.* (1991b) showed that selection of silver foxes for tame behaviour resulted in changes in the pituitary-adrenocortical system as well as in gonadal hormone regulation.

One particular neurotransmitter has received overwhelming attention in the field of aggression research: serotonin (5-hydroxytryptamine, 5-HT). Serotonin plays a central role in the molecular biology of the mind. Some authors argue that most of the substances mentioned above exert their effect on aggression through the serotonergic system (Chiavegatto and Nelson 2003; Nelson and Chiavegatto 2001). There is also substantial evidence that 5-HT is involved in canine aggression.

## Serotonin

Serotonin is an ancient neurotransmitter that is found in primitive life forms such as flatworms (Venter *et al.* 1988). Serotonergic neurons project from the raphe nuclei in the brain stem and innervate areas throughout the central nervous system. These areas include the hypothalamus, cortex, hippocampus, amygdala, and striatum; areas known to regulate cognition and emotion. Serotonergic neurons are also highly bifurcated, enabling them to influence the function of several regions of the central nervous system simultaneously (Lucki 1998). The effects of 5-HT are mediated through at least 14 receptor subtypes (Hoyer *et al.* 1994; 2002). We have summarised the neurochemistry of 5-HT in Figure 2.

The role of 5-HT in normal and abnormal behaviour has been studied extensively. Serotonin influences physiological and behavioural processes such as thermoregulation, sleep-wake cycle, anxiety, aggression, sexual behaviour, impulse inhibition, pain sensitivity, and food intake (Lucki 1998). The 5-HT system is the target of pharmacological treatment of various mental disorders, including depression, anxiety disorders, obsessive-compulsive disorder, and schizophrenia (Cravchik and Goldman 2000). Serotonin also plays a role in

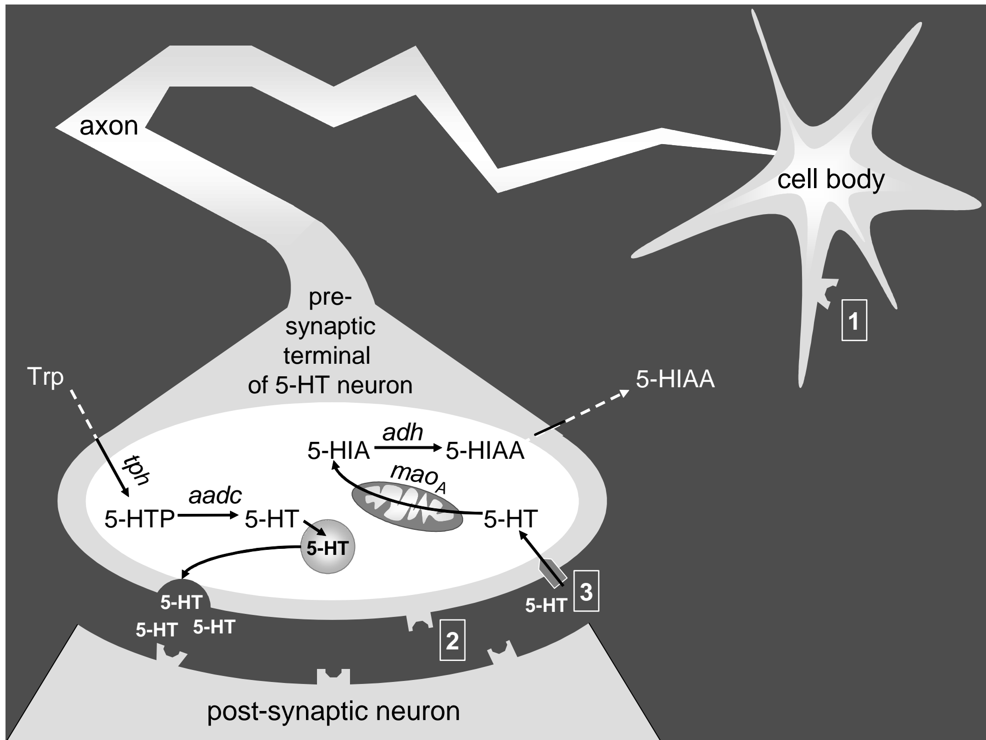


**Figure 2.** Schematic summary of the neurochemistry of serotonin (5-HT). The essential amino acid tryptophan (Trp) is transported from the plasma into the brain. Trp is then converted into 5-HT in two steps. In the first step, Trp is hyroxylated to form 5-hydroxytryptophan (5-HTP). This reaction is catalyzed by the rate-limiting enzyme tryptophan hydroxylase (*tph*). In the second step, 5-HTP is rapidly carboxylated to form 5-HT. This is catalyzed by aromatic L-amino acid decarboxylase (*aadc*). Most of the 5-HT is stored in vesicles. Upon excitation, 5-HT is released into the synapse by exocytosis. Somatodendritic (5-$HT_{1A}$; marked with 1 in the figure) and presynaptic (5-$HT_{1B}$ or 5-$HT_{1D}$; marked with 2 in the figure) autoreceptors regulate this release. The serotonin transporter (marked with 3 in the figure) terminates the effects of released 5-HT through re-uptake into the 5-HT neuron. In the neuron, the mitochondrial enzyme monoamine oxidase ($MAO_A$) degrades 5-HT into 5-hydroxyindoleacetaldehyde (5-HIA). This is then rapidly oxidised by aldehyde dehydrogenase (*adh*) to form 5-hydroxyindoleacetic acid (5-HIAA). 5-HIAA then diffuses into the cerebrospinal fluid. (See Feldman *et al.* 1997 for a detailed description of 5-HT neurochemistry.)

early brain development and adult neurogenesis (Azmitia and Whitaker-Azmitia 1997; Gould 1999; Lauder 1990; 1993; Sodhi and Sanders-Bush 2004). The 5-HT system functions as a "master control system", integrating complex brain functions through its interactions with other neurotransmitter systems (Lesch and Merschdorf 2000).

Scientists have assessed the role of 5-HT in aggressive behaviour using various approaches, including pharmacological studies, analyses of cerebrospinal fluid, dietary manipulations of tryptophan, imaging studies, knockout mutations in mice, and mutation analyses. These studies provide a large body of evidence that the 5-HT system is a major modulator of aggressive behaviour (see for instance Berman and Coccaro 1998; Feldman *et al.* 1997; Gingrich and Hen 2001; Lesch and Merschdorf 2000; Lucki 1998; and Olivier *et al.* 1995 for reviews).

The 5-HT system has also been studied in canine aggression. In 1996, Reisner and colleagues presented a study of cerebrospinal fluid (CSF) contents in dominant-aggressive dogs. The CSF levels of metabolites of neurotransmitters are considered to reflect the activity of the corresponding neurotransmitter systems. A rich literature deals with possible associations between low CSF concentrations of the major metabolite of 5-HT, 5-hydroxyindole acetic acid (5-HIAA), and aggressive behaviour in humans, non-human primates, and rodents (e.g. Berman and Coccaro 1998; Fairbanks *et al.* 2001; Linnoila *et al.* 1993; Mehlman *et al.* 1994; Roggenbach *et al.* 2002). Reisner *et al.* (1996) measured the CSF level of 5-HIAA, homovanillic acid (HVA), and 3-methoxy-4-hydroxy phenylglycol (MHPG), which are the major metabolites of serotonin, dopamine, and norepinephrine, respectively. Reisner *et al.* compared CSF of 21 dominant aggressive dogs of various breeds and 19 non-aggressive controls. The diagnosis of dominance aggression was based on an interview with the dog owner. Dogs were included in the aggressive group if they had exhibited aggressive behaviour toward family members in the context of a dominance conflict. CSF 5-HIAA and HVA concentrations were significantly lower in dominant-aggressive dogs than in controls. There was no significant difference in CSF MHPG concentrations between the two groups. Within the aggressive group, dogs that attacked without warning had lower concentrations of CSF 5-HIAA and HVA than dogs that warned. The differences in 5-HIAA were maintained after controlling for breed and age. By contrast, the HVA differences might have been breed-dependent, because 63% of the control dogs were Beagles and the Beagles had higher HVA than the other control dogs. In spite of the poor matching of cases and controls, the results of this study suggest an association between reduced 5-HT function and canine aggression.

The 5-HT system was also addressed in the large selection experiment for tameness in silver foxes (Popova *et al.* 1991b). The researchers compared components of the brain 5-HT system in 34 silver foxes selected for tameness (i.e. reduced defensive response to humans) for about 20 generations and 29 unselected wild silver foxes bred in captivity over the same time span. Tame

foxes had higher 5-HT levels in hypothalamus and midbrain and higher 5-HIAA content in midbrain, hypothalamus, and hippocampus. Tryptophan hydroxylase activity was increased in midbrain and hypothalamus of tame foxes compared to the wild ones. In contrast, monoamine oxidase A ($MAO_A$) activity was decreased in domesticated foxes. The density of 5-$HT_{1A}$ receptors in hypothalamic membranes was lower in tame silver foxes than in their wild counterparts. Selection for tameness in rats resulted in similar changes in the 5-HT system, supporting the hypothesis that the brain 5-HT system is involved in domestication (Naumenko *et al.* 1989; Popova *et al.* 1991a). This involvement might be established through the role of 5-HT in the regulation of the pituitary-adrenal system, which is involved in stress responses.

Single-photon emission tomography (SPET) has been used to study the density of 5-$HT_{2A}$ receptors in the brains of impulsive aggressive dogs (Peremans *et al.* 2003; 2005). SPET is an imaging technique that offers the opportunity to investigate neuroreceptor binding directly *in vivo*. Peremans and colleagues used a radioligand to visualise 5-$HT_{2A}$ receptors in the canine brain. The 19 dogs in the aggressive group were of various breeds and had a history of aggression towards people familiar to them. The aggressive behaviour was unpredictable (i.e. without classical warning signals and in unforeseen circumstances) and the provoking stimulus was not in proportion to the intensity of the attack. The reference group consisted of 12 Shepherd-type dogs which never showed this behaviour. Like in the CSF studies of Reisner and colleagues (1996), questionnaires were used to obtain behavioural information. A higher 5-$HT_{2A}$ binding index was found in the cortex of impulsive aggressive dogs compared to the reference dogs. In a follow-up study, the researchers treated nine of the impulsive aggressive dogs with citalopram hydrobromide, a selective serotonin reuptake inhibitor. The 5-$HT_{2A}$ receptor binding index was significantly reduced after treatment with citalopram hydrobromide. The dog owners were asked whether the behaviour of the dog had improved because of the pharmacological therapy. Clinical improvement in behaviour of the dogs correlated with a reduced 5-$HT_{2A}$ binding index. These findings support the hypothesis that the 5-HT system is involved in canine (impulsive) aggression.

Badino and colleagues (2004) measured adrenergic and serotonergic receptor concentrations in brains of eight aggressive and eight non-aggressive male dogs. Their methods allowed for the detection of two main types of 5-HT receptors: low-affinity and high-affinity 5-HT receptors. They demonstrated an increased concentration of the low affinity type in all investigated brain areas in aggressive dogs compared to non-aggressive controls (frontal cortex; hippocampus; thalamus; hypothalamus). The high affinity receptors were only

increased in thalamus and hypothalamus. In addition, they reported a significant decrease in beta-adrenergic receptor levels in the frontal cortex, hippocampus, and thalamus of aggressive dogs compared to controls. This suggests that the serotonergic and the catecholaminergic system are both involved in canine aggression.

The role of 5-HT in canine aggression is further supported by clinical evidence. Several behavioural experts advise pharmacological intervention in the 5-HT system for the treatment of aggression in dogs (e.g. Dodman 1998; Landsberg 2004). This is supported by a small study of Dodman *et al.* (1996a), who studied the effects of treatment with fluoxetine or a placebo in nine dogs diagnosed with owner-directed dominance aggression. Fluoxetine is a selective serotonin reuptake inhibitor. It was found that fluoxetine significantly reduced owner-directed dominance aggression after three weeks of treatment. In another type of study, DeNapoli *et al.* (2000) evaluated the effect of dietary protein content and tryptophan supplementation on canine dominance and territorial aggression. Tryptophan is the precursor of 5-HT (Figure 2) and tryptophan depletion has been used by some researchers to experimentally manipulate serotonin levels in human subjects (reviewed by Krakowski 2003). DeNapoli and colleagues asked owners to score the behaviour of their dog while the dog was on one of four different diets. Dogs that were fed unsupplemented high-protein diets obtained the highest dominance aggression scores. Dogs that were fed tryptophan-supplemented low-protein diets obtained low scores for territorial aggression.

## Genetics of canine aggression

### Genetic model

Genes encode proteins that are important for the development and regulation of the neural pathways that regulate behaviour. Like most other behaviours, aggression is likely to be under polygenic control. In a study of aggression in Dutch Bernese Mountain Dogs, increasing aggression grade of parents gave rise to an increase in grade of aggression of progeny (van der Velden *et al.* 1976). This is indicative of a polygenic trait. Although the normal range of variation in aggression in dogs is probably regulated by multiple genes and environmental influences, certain dog families may segregate a gene with a major effect on aggressiveness. An example of a single gene with a major effect on aggression has been described in a Dutch human family by Brunner *et al.* (1993a; 1993b). Male members of this family showed impulsive violent

behaviour, including arson, rape, and murder; and they were mentally retarded. The researchers discovered a point mutation in the *$MAO_A$* gene of the subjects. The $MAO_A$ enzyme catalyzes the degradation of 5-HT, dopamine, and norepinephrine. The aggressive males produced no $MAO_A$ enzyme because the mutation caused a premature stop codon. This illustrates the idea of Plomin (1990) that *any one of many genes can disrupt development, but the normal range of behavioural variation is likely to be orchestrated by a system of many genes, each with small effect, as well as environmental influences.*

## Evidence for a genetic basis of canine aggression

The relative importance of genetic and environmental influences on behaviour is a fundamental question in behavioural genetics. Family designs, adoption designs, and twin designs have been used to address this question in human behavioural genetics (Boomsma *et al.* 2002; Plomin 1990). Such studies have shown that there is a significant genetic influence on the majority of behaviours examined, including aggressive behaviour (Coccaro *et al.* 1997; Hamer 2002; Plomin 1990; Rhee and Waldman 2002; Slutske 2001). Genetic analyses of animal behaviour have traditionally been strain studies (e.g. comparing the aggressiveness of various strains of mice; reviewed by Popova *et al.* 1993), selection studies (e.g. the selective breeding program for offensive aggression resulting in short attack latency and long attack latency mice; reviewed by Miczek *et al.* 2001), or population-based quantitative genetic studies (e.g. studies of feather pecking behaviour in laying hens, Rodenburg *et al.* 2003). Like the studies of human subjects, the animal studies have demonstrated that the tendency to behave aggressively is heritable (Miczek *et al.* 2001).

### *The result of dog breeding*

Humans have created dog breeds by artificial selection. Some breeds have intentionally been selected for aggressiveness. For instance, Rottweilers, Dobermanns, German Shepherds, and Akitas have been selected for stranger-directed aggression. American Pit Bull Terriers have been selected for fighting purposes (Lockwood and Rindy 1987). Traits can only be selected for if they are heritable. Interbreed differences in behavioural traits therefore point to a heritable basis of the traits concerned. As Ostrander and Giniger (1997) noted, *it is apparent from even casual observation that the behaviours and much of the personality of a dog can be characteristic of its breed, rather than being strictly individual. Consider the Pointer that points, the Draft dog that pulls, and the Bloodhound that tracks. A Border*

*Collie raised away from a farm will still demonstrate the classic herding behaviours of circling, crouching, and eye when appropriately prompted.* There is ample anecdotal evidence that dog breeds differ in aggressiveness (e.g. American Pit Bull terriers; Lockwood and Rindy 1987). The results of several studies also provide scientific evidence for breed differences in aggressiveness. We will discuss three types of studies: retrospective studies of clinical data, surveys, and behavioural tests.

Several authors have listed breed characteristics of dogs referred to behavioural clinics for aggression problems (e.g. Borchelt 1983, Blackshaw 1991, Beaver 1993). They concluded for instance that dominance aggression was more likely in English Springer Spaniels. German Shepherd dogs seemed to predominate in protective aggression. However, such statistics are likely to be biased by the proportion of the breed in the general dog population. Landsberg (1991) has tried to circumvent this problem by comparing the behaviour cases with breed registrations. He concluded that the number of Springer Spaniels, Wheaten Terriers, Dobermanns, Old English Sheepdogs, and Dalmatians presented for aggression was out of proportion to their breed registration numbers. The reliability of these numbers is questionable, even though they were corrected for the proportion of the breed in the general dog population. An example of a source of bias is that clients with particular dog breeds might seek help quicker than clients with other breeds (Overall 1997). The same objections apply to breed-specific bite rates. Both the number of bites attributed to a breed and the number of animals in that breed are required to compute accurate breed-specific bite rates. These data are usually not available. Lockwood and Rindy (1987) have written a critical commentary on this topic. We can conclude at most that the retrospective studies of clinical data provide tentative evidence for breed differences in aggressiveness.

It is interesting to note that the English Springer Spaniel is mentioned as an aggressive breed by many authors (Borchelt 1983, Beaver 1993; Landsberg 1991; Takeuchi 2001). Recently, a mail survey has been performed among 2400 randomly selected owners of American Kennel Club-registered English Springer Spaniels (Reisner *et al.* 2005). A history of owner-directed aggression was reported in 510 dogs. The researchers analysed four-generation pedigrees of the dogs. One particular popular sire was significantly associated with owner-directed aggression, suggesting a genetic predisposition.

Frequently cited evidence for breed differences in aggressiveness comes from a number of surveys. Hart and Miller (1985) interviewed a group of randomly selected veterinarians and dog obedience judges. Their objective was to obtain behavioural profiles of dog breeds that would allow prospective dog owners to choose a breed by its behaviour (Hart and Hart 1988). The

veterinarians and judges were asked to rank breeds from the lowest to the highest level of expression of 13 behavioural traits. The behavioural traits included snapping at children, watchdog barking, aggression to dogs, dominance over owner, and territorial defence. They found significant differences between breeds for all traits. For instance, Rottweilers and Dobermanns scored in the highest decile for watchdog barking, whereas Saint Bernards and Basset Hounds were in the lowest decile. Bradshaw and Goodwin (1996; 1999) replicated this study in the United Kingdom. Again, all aggression-related questions produced significant separations between the breeds. Of the 36 breeds in common between the studies, 24 had similar behavioural characteristics. However, as Overall (1997) also pointed out, the conclusions from these surveys should be treated with caution because the results are probably biased by shared stereotypic beliefs about behavioural characteristics of breeds.

The most objective evidence for breed differences in aggressiveness has been obtained with behavioural tests. Scott and Fuller (1965) performed an extensive experiment lasting 13 years at Jackson Laboratory in Bar Harbour, Maine. The aim was to determine the influence of hereditary differences on behaviour. They studied the behaviour of hundreds of purebred and hybrid dogs from birth to one year of age. Five breeds were used: Basenjis, Beagles, American Cocker Spaniels, Shetland Sheepdogs, and Wire-haired Fox Terriers. They found behavioural differences between the breeds in the majority of their behavioural tests. For instance, there were breed differences in playful aggression and dominance, with Wire-haired Terriers at the top (i.e. most aggressive) and Cocker Spaniels at the bottom of the scale. The Wire-haired Terriers developed a large number of dominance relationships before 15 weeks of age, other breeds being slower. They concluded that the Terriers were the most aggressive, the Basenjis and Shelties next, and the other two breeds the least. The Fox Terrier puppies also consistently "ganged up" on group members. These attacks were so serious that victims had to be removed in order to prevent serious injury.

Scott and Fuller (1965) derived their purebred stocks from a few closely related individuals. The behaviour of this small number of individuals is not necessarily representative of the entire breed. We can therefore draw only limited conclusions from the breed comparisons. However, Scott and Fuller also performed crosses between Basenjis and Cockers Spaniels. They studied the inheritance of traits by comparing performance of pure-bred dogs, hybrids and backcrosses in the behavioural tests. Their conclusion was that there are average breed differences for most traits. However, they also cautioned against the idea of breed stereotypes because the variation within breeds was large.

Two more recent studies have also used behavioural tests to evaluate breed differences. Wilsson and Sundgren (1997) compared behavioural test scores of German Shepherd dogs and Labrador Retrievers. German Sherpherds scored higher for sharpness (defined as the tendency to react with aggression) and defence drive (the tendency to defend itself or its handler). Svartberg (2006) compared the behaviour of 31 breeds using data from the Swedish dog mentality assessment test from 13097 dogs. There were significant differences between the breeds in all investigated traits, including aggressiveness. However, like Scott and Fuller (1965), Svartberg (2006) reported large variation within the breeds. This is illustrated in Figure 3.

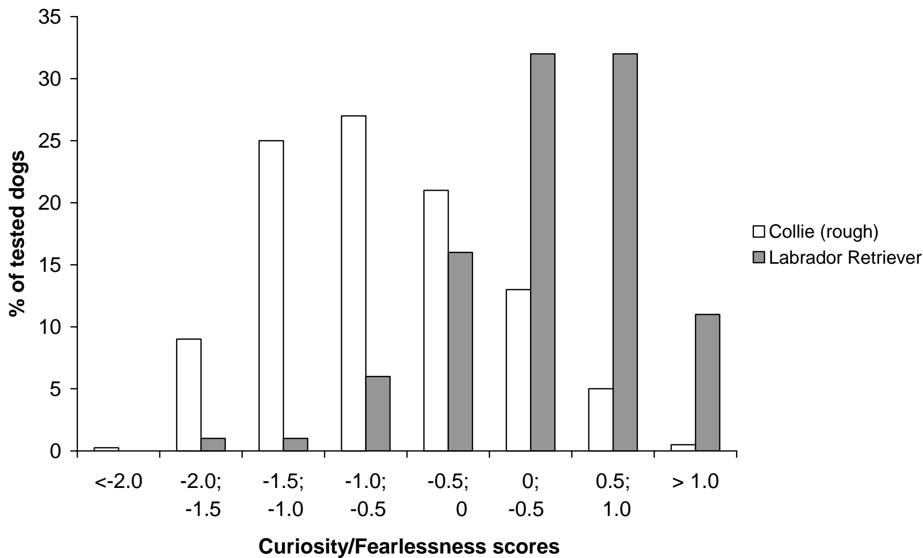


**Figure 3.** This figure shows a result of the study of breed-typical behaviour by Svartberg (2006). The distribution of curiosity/fearlessness scores is shown for two dog breeds: Collie and Labrador Retriever. Labradors were the highest ranking breed for this trait and Collies ranked the lowest. It is clearly visible that, although there is a lot of variation within the breeds, the means of the distributions of the breeds are shifted with respect to each other. (Figure reprinted from Svartberg 2006 with permission from Elsevier.)

We can conclude that there is ample scientific evidence that some breeds have a higher tendency to behave aggressively than others. This is likely to be caused by genetic differences between the breeds. However, it is also obvious from the studies that there is high individual variation in aggressiveness within breeds. This within-breed variance is likely to be the result of genetic variation as well. Svartberg (2006) noted that rapid changes in canine behavioural traits seem to be possible in few generations, stressing the importance of behavioural considerations in dog breeding.

a

b

**Figure 4.** Silver fox selected for aggressiveness (a) and silver fox selected for tameness (b) at the Institute of Cytology and Genetics in Novosibirsk.

*Selection studies*

The experimental studies of silver foxes at the Institute of Cytology and Genetics in Novosibirsk demonstrate more directly that aggression can be manipulated by selection (Trut 1999; 2001). Foxes were selected for tameness (i.e. lack of fearful or aggressive behaviour towards human experimenters) for more than 35 generations at this institute. The response to human presence was the only selection criterion. Foxes that were eager to establish human contact, whimpering to attract attention and sniffing and licking experimenters like dogs do, appeared already in the sixth generation (Trut 1999; see Figure 4).

The tameness resulted from genetic selection alone because the foxes were not trained (Popova *et al.* 1991b). By the 20th generation, 35% of the foxes behaved like dogs. The scientists also succeeded in breeding a colony of foxes with enhanced aggressive behaviour (Gulevich *et al.* 2004). These animals were selected for an aggressive and fearful response to the experimenter approaching their cage.

*Heritability estimations*

Heritability ($h^2$) describes the contribution of additive genetic variance to phenotypic variance (Bourdon 1997b; Nicholas 2003). Several reviews discuss heritability estimations of behavioural traits in dogs (Houpt and Willis 2001; Mackenzie *et al.* 1986). Some early studies of aggression-related traits in police or military dogs failed to produce $h^2$ estimates higher than zero (Barlett 1976; Pfleiderer-Hogner 1979; Reuterwall and Ryman 1973; Willis 1976). However, this is probably due to methodological problems (Houpt and Willis 2001; Mackenzie *et al.* 1986; and personal communication with A.-E. Liinamo). Three recent studies obtained $h^2$ estimates between 0.06 and 0.33 for aggression scores in behavioural tests (see Table 2). It thus seems that the heritability of aggression is low but significant in the general dog population. However, the

**Table 2.** Heritability ($h^2$) estimations of canine aggression-related traits in three studies.

| **Trait** | **Breed*** | **$h^2$** | **Reference** |
|---|---|---|---|
| attacking during defence competitions | BSD | 0.14 | Courreau and Langlois (2005) |
| biting during defence competitions | BSD | 0.16 | |
| guarding during defence competitions | BSD | 0.14 | |
| dominant-aggressive behaviour in the Campbell test | ECS | 0.33 | Pérez-Guisado *et al.* (2006) + |
| aggression in response to sudden appearance in DMA# | GSD | 0.10 | Saetre *et al.* (2006) |
| | R | 0.10 | |
| aggression towards ghost in DMA# | GSD | 0.12 | |
| | R | 0.06 | |

* BSD = Belgian Shepherd dog, mainly of the Malinois breed; ECS = English Cocker Spaniel; GSD= German Shepherd dog; R= Rottweiler
# DMA = dog mentality assessment test
+ This study had a small sample size (n=51), so the results are of questionable reliability.

legitimacy of this conclusion depends on the validity of the measures of aggression. This is a topic that warrants further research. Finally, it is important to note that heritability estimations for fear are generally much higher than those for aggression (Houpt and Willis 2001). This is relevant because aggression can be motivated by fear.

**Aggression genes**

Gingrich and Hen (2001) have reviewed studies of serotonergic knockout mice. Increased aggression has been reported in mice lacking the gene encoding serotonin receptor 1B (*htr1B*; Saudou *et al.* 1994; see Figure 2 for the function of the gene product in 5-HT metabolism and neurotransmission) and in mice lacking the gene encoding $MAO_A$ (Cases *et al.* 1995; Figure 2). The latter substantiates the findings of Brunner and colleagues (1993a; 1993b) of a null mutation in the $MAO_A$ gene in a violent family. Serotonin receptor 1A knockout mice are extremely anxious (Heisler *et al.* 1998; Parks *et al.* 1998; Ramboz *et al.* 1998). Several other authors have reviewed aggressive knockout mice without limiting themselves to the 5-HT system (Miczek *et al.* 2001; Nelson and Chiavegatto 2001; Tecott and Barondes 1996). In addition to *maoA* and *htr1B* knockouts, increased aggression has been found in mice lacking the gene encoding adenosine 2a receptor (Ledent *et al.* 1997), adrenergic alpha 2C receptor (Sallinen *et al.* 1998), beta estrogen receptor (Ogawa *et al.* 1999), breakpoint cluster region (Voncken *et al.* 1998), enkephalin (Konig *et al.* 1996), neural cell adhesion molecule (Stork *et al.* 1999), neuronal nitric oxide synthase (Huang *et al.* 1993; Nelson *et al.* 1995), or neutral endopeptidase (Fisher *et al.* 2000). Conversely, scientists have found decreased aggression in mice lacking the gene encoding alpha-calcium-calmodulin kinase II (Chen *et al.* 1994; Silva *et al.* 1992), alpha estrogen receptor (Ogawa *et al.* 1997), histamine 1 receptor (Inoue *et al.* 1996; Yanai *et al.* 1998), endothelial nitric oxide synthase (Demas *et al.* 1999), glutamic acid decarboxylase 65 amino acids (Stork *et al.* 2000), neurokinin 1 receptor (De Felipe *et al.* 1998), regulator of G protein signalling (Oliveira-dos-Santos *et al.* 2000), or knockouts for both alpha and beta estrogen receptors (Ogawa *et al.* 2000). In oxytocin knockout mice both increased (Winslow *et al.* 2000) and decreased (DeVries *et al.* 1997) aggressiveness have been reported. Some of these gene products are connected with the 5-HT system. For instance, the enzyme alpha-calcium-calmodulin kinase II is required for the activation of tryptophan hydroxylase, the rate-limiting step in 5-HT synthesis (New *et al.* 1998; see also Figure 2). Whole brain 5-HIAA is elevated in adrenergic alpha 2C receptor knockout mice, whereas whole brain 5-HT is decreased (Sallinen *et al.* 1998).

Takeuchi and Houpt (2003) listed polymorphic genes that have been associated with temperament or mental disease in humans. In addition to the $MAO_A$ gene, this list includes the gene encoding the serotonin receptor 1B and tryptophan hydroxylase as genes involved in aggression. Other studies demonstrated a correlation between dopamine D3 receptor polymorphisms and aggressive behaviour (e.g. Retz *et al.* 2003).

There have been no studies linking specific genes to aggression in dogs. Two research groups in Japan have studied allelic variation of genes involved in neurotransmission in dogs. A group at Gifu University has published several studies of the canine dopamine D4 receptor gene (Inoue-Murayama *et al.* 2002; Ito *et al.* 2004; Niimi *et al.* 1999; 2001). A polymorphism in this gene may be associated with the personality trait of novelty seeking in humans (Benjamin *et al.* 1996; Ebstein *et al.* 1996). The researchers studied allele frequencies of the polymorphism in 1535 unrelated dogs of 23 breeds. Aggressive breeds tended to have higher frequencies of three specific alleles. However, the behavioural characteristics of the breeds were derived from expert ratings similar to those in the studies of Hart and Miller (1985). As we noted before, such ratings are probably biased. In order to demonstrate an association between the polymorphism and aggression, phenotypes of individual genotyped dogs should be included in the study.

A list of polymorphic genes that could serve as candidate genes for canine temperament is being developed by Masuda *et al.* (2004b). To date, they have identified polymorphisms in the canine genes encoding catechol O-methyltransferase (Masuda *et al.* 2004a), serotonin receptor 1B (Masuda *et al.* 2004b), monoamine oxidase B (Hashizume *et al.* 2005), tyrosine hydroxylase (Takeuchi *et al.* 2005), and dopamine β-hydroxylase (Takeuchi *et al.* 2005). All of these genes are involved in neurotransmission and they have been suggested to play a role in personality traits or mental disorders in humans. The researchers genotyped the polymorphisms in five dog breeds. Genotype and allele frequencies differed between the breeds for the majority of the polymorphisms.

It has been suggested that the breed differences in allele frequencies are related to breed differences in behaviour (Houpt and Willis 2001). However, many markers display breed differences (Parker *et al.* 2004), so a study of the association between these polymorphisms and canine temperament should include behavioural information of individual genotyped dogs. The work of the Japanese researchers provides a good starting point for such candidate gene studies.

## Dog genome resources

In recent years, the field of canine genomics has made giant leaps forward (see Sutter and Ostrander 2004 for a review). Two canine whole-genome sequences are now available (Kirkness *et al.* 2003; Lindblad-Toh *et al.* 2005), providing 1.5-fold and 7.5-fold coverage of the genome. We have access to a 900-kb resolution genetic map of the dog containing 4249 markers (Breen *et al.* 2004; Guyon *et al.* 2003). In addition, comparing the two whole-genome sequences and resequencing in other breeds has generated a canine SNP map containing more than 2.5 million SNPs mapped to the draft genome sequence (Lindblad-Toh *et al.* 2005). The recent studies of the canine genome have confirmed its promise for mapping disease genes. For instance, Sutter and colleagues (2004) showed that linkage disequillibrium (LD) in dogs extends 10 to 100 times longer distances than does LD in even the most isolated human populations. The extent of LD greatly reduces the number of markers required for genome-wide association studies. In other words: the harvest can start!

CHAPTER 2

# Phenotype analysis

# 2.1
# Phenotyping of aggressive behaviour in Golden Retrievers with an aggression test

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This section has been adapted from "Behavior genetics of canine aggression: behavioral phenotyping of Golden Retrievers by means of an aggression test"
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## Abstract

Molecular genetic analysis of complex traits like aggression strongly depends on careful phenotyping of individuals. When studying canine aggression, the information provided by owners of the dogs may not be detailed and reliable enough for this purpose. Therefore we subjected 83 Golden Retrievers, both aggressive and non-aggressive individuals, to a behavioural test. The tests were analysed using an ethogram, resulting in a behavioural profile for each dog. In this section, we describe three methods for converting these profiles into a behavioural phenotype. The usefulness of the methods is evaluated by comparing the test results with information provided by dog owners. In addition, we evaluated the hypothesis that a lowered threshold for aggressive behaviour in general is present in the aggressive Golden Retrievers. Future research will need to reveal whether the methods meet the high standards that are necessary for studying complex traits.

## Introduction

Biting incidents with dogs pose a considerable problem in countries all over the Western world. Each year about 240 people are hospitalised as a consequence of dog bites in the Netherlands (Mulder 1991; Schellart and den Hertog 1998; Toet and den Hertog 2000). Aggression is a common reason for euthanasia of dogs (Mikkelsen and Lund 2000). Studying the aetiology of canine aggression is therefore important for both human and canine welfare (Hunthausen 1997; Rossman *et al.* 1997; Rusch *et al.* 2000). Canine behavioural disorders are also interesting because they can serve as a model for human mental disorders. Dog behaviour may be a valid model for human behaviour because both dogs and humans show within group competition as well as cooperation (Overall 2000).

Aggression is a complex trait in any species. It is under polygenic control and environmental factors play a role in its development (Enserink 2000; Mackenzie *et al.* 1986; Tecott and Barondes 1996). The nature, relative importance, and interaction of these genetic and environmental influences are poorly understood. Our studies of the aetiology of canine aggression focus on genetics. The low genetic heterogeneity within dog breeds implies that only a limited number of the genes that influence aggression will be functionally polymorphic within a breed (van Oost *et al.* 2002). We study dogs of the Golden Retriever breed. Golden Retrievers are usually friendly pets, but some are very aggressive (Edwards 1991; Galac and Knol 1997; Heath 1991; Knol and Schilder 1999). It is likely that a genetic cause is involved because aggressive behaviour seems to occur more often in certain Golden Retriever family groups (Knol *et al.* 1997).

Molecular genetic analysis of complex traits strongly depends on careful phenotyping of individuals. The *diagnostic and statistical manual* (DSM) and the *international classification of diseases* (ICD) are often applied in studies of the genetics of human mental disorders. No such instrument is available for studies in dogs. Questionnaires for dog owners have been applied in some studies of canine behavioural problems. However, owners are not always skilled in observing behaviour and using their opinions might lead to biased results (Galac and Knol 1997; Hart 1995; van der Borg *et al.* 1991). Moreover, the information provided by owners is likely to reveal only a limited number of phenotypic classes (for example "aggressive" and "non-aggressive"). A more detailed classification might be required for molecular genetic studies. We therefore studied the possibilities of using a behavioural test as an objective and detailed method for assessing behavioural phenotypes in dogs. Several canine behavioural tests have been described. Van der Borg *et al.* (1991)

described a test for dogs in animal shelters, which aimed to prevent bad matches between new owners and dogs. They correctly predicted 75% of the problem behaviours that a dog would show in the future with the test. Netto and Planta (1997) published a test that could be used for excluding aggressive individuals from breeding. The aggressive tendencies of the dog were evaluated in 43 subtests. They concluded that their test was a useful instrument for assessing these tendencies. In addition to these two tests, numerous other tests for dog behaviour exist, but few of these were scientifically validated. We used a shortened version of the test described by Netto and Planta for phenotyping Golden Retriever behaviour.

The test can only be useful for genetic studies if the variety of behaviours observed during the test is translated into a certain measure. Two main approaches are possible for this translation, each with its specific underlying hypothesis about the aetiology of aggression. The first approach adds behaviours observed during various subtests and does not take into account that the subtests offer different types of stimuli. Here, the underlying hypothesis is that the aggressive Golden Retrievers have a lowered threshold for aggressive behaviour under various circumstances. This hypothesis is supported by Netto and Planta (1997), who suggested that highly aggressive behaviour during their test was the result of a genetically based tendency for aggression. The second approach analyses dog behaviour during classes of subtests with similar stimulus situations. The hypothesis underlying this approach is that aggression can be subdivided into classes based on the nature of stimuli eliciting the behaviour and that these classes are controlled by different genetic mechanisms. Literature provides some evidence for this assumption (Blackshaw 1991; Borchelt 1983; Moyer 1976; Popova *et al.* 1993; Voith 1984; Wright and Nesselrote 1987). It is not clear which of the two approaches is the best because the nature of the genetic basis of aggression is still poorly understood.

We analysed the behaviour of 83 Golden Retrievers, 59 aggressive and 24 non-aggressive, during the test using an ethogram. The analyses are based on the hypothesis that a lowered threshold for overall aggressive behaviour is present in the aggressive dogs. We thus added data from various subtests. Within this approach, there are various ways of converting the test results into a behavioural phenotype. We will discuss three methods. Although the impression of the owner was considered to be moderately reliable, the usefulness of the methods was evaluated by comparing the phenotypic scores with owner-provided information about the dogs.

## Methods

### Subjects

The study group consisted of 83 Golden Retrievers, 55 of which were purebred with a pedigree. All Goldens were privately owned dogs, and the majority (82%) still lived with their first owners. There were 49 males (18 castrated) and 34 females (16 castrated) in the study group. The mean age of the dogs at the time of testing was 3.3 years; three were juveniles (8 or 9 months), 8 dogs were sub-adults (9-18 months), 67 dogs were adults (18 months-7 years), and 5 were old dogs (7 years or older). Fifty-three of the 83 dogs were referred to behaviour experts at the Utrecht University Companion Animal Clinic because of their aggressiveness. We consequently traced 30 family members (mainly siblings) of a number of these Goldens. Although none of these relatives had ever bitten people or another dog, 6 of them showed a problematic level of aggressiveness according to their owner. In none of the dogs was a medical problem likely to be the origin of the aggressive behaviour and none of the dogs received medication.

### Personal interview

We collected information about aggressive behaviour of the dogs during a personal interview with the dog owners. Based on this information, we classified dogs as "owner-acknowledged non-aggressive" if the owner declared that the dog had never attacked, bitten or showed excessive growling behaviour towards either a dog or people. All other dogs were classified as "owner-acknowledged aggressive".

### The aggression test

The aggression test consisted of 22 subtests. The majority (19) of these were selected from the Netto and Planta test (1997) because they had high aggression-eliciting power. Two less threatening subtests (subtests 4 and 5) were included in order to let the dog acclimatise to the testing room and to make the test more acceptable for the owner. A new subtest, using a dog mask (subtest 21), was added. Tests were performed in the facilities for dog research at Utrecht University, which were previously described (Netto and Planta 1997, see also Figure 1). The tests were performed by three people: two testers (one male and one female) and a cameraman. The testers verbally reported behavioural elements if these were unlikely to be visible or audible on the video

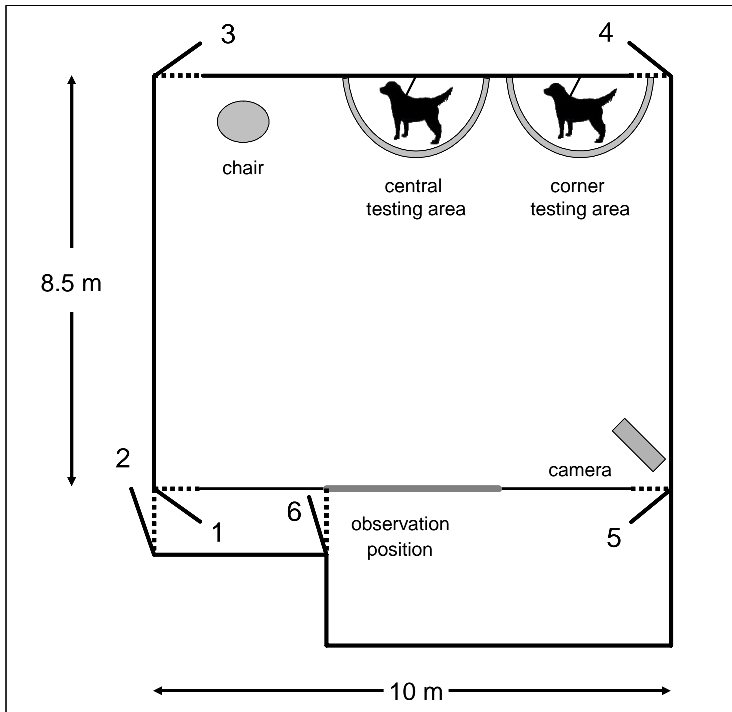



**Figure 1.** Overview of the indoor testing facilities. Doors are numbered from 1 to 6. Subtests 6-12 and 17-22 were performed in the central testing area, whereas subtests 13-16 were performed in the corner testing area. If the owner was present, he or she would either sit on the chair adjacent to the central area, or stand next to the dog in the corner testing area. The owner left and re-entered the room through door 3.

tape. All subtests lasted twenty seconds, except for subtest 4 and 5. Pauses between the subtests were kept as short as possible. The subtests are:

1. Two testers approach the dog-owner's car containing the dog and both stare at the dog and knock on the car window. The owner is out of sight of the dog during this subtest.

After the first subtest, the owner walks the dog up and down with a leash outdoors and demonstrates the obedience of the dog to the basic commands "sit", "down", and "come".

2. Confrontation with two free-running barking stimulus dogs behind a fence (length 20 m). The owner walks the dog with a leash once along the fence and back again at a distance of 1 m from the fence. One of the testers is also standing behind the fence.
3. Confrontation of the dog (in the absence of the owner) with a barking dominant stimulus dog behind a fence. A tester holds the Golden on the leash. Again, one of the testers is standing behind the fence.

After subtest 3 the dog is transferred to the adjacent test room (Figure 1), where all other subtests are carried out. The dog is given the opportunity to explore the test room prior to subtest 4.

4. The owner plays tug-of-war with the Golden for 1 minute using an unfamiliar toy (O'Farrell 1986; van der Wijk and Klasen 1981).
5. A tester plays tug-of-war with the dog for 1 minute using the same toy as in subtest 4. The owner is sitting on the chair in the test room.

The owner attaches the dog to a hook with a leash in the central testing area.

6. The owner squeezes the skin on the groins of the dog rather tightly.
7. Using an artificial hand, a tester pulls away the feeding bowl of the dog while the dog is eating (dry dog food). The artificial hand is a plastic natural-looking model of a hand, with a stick attached to it. The stick is covered with a sleeve to hide the real hand of the tester. The bowl is pulled away and pushed back to its original position repetitively. At the start of this subtest, the owner places the bowl in the right position and he/she then takes a seat on the chair next to the dog (van der Borg *et al.* 1991).
8. Using his/her own hand or an artificial one, the owner pulls away and pushes back the feeding bowl of the dog while it is eating.

The owner now leaves the room through door 5 and subtests 9 through 12 are performed in the absence of the owner.

9. The male tester repeatedly opens an umbrella with an automatic opening device in front of the dog.
10. The female tester, dressed as a strange-looking woman walking with a stick, approaches the dog, tries to pet the dog using the artificial hand and speaks in a strange high piercing voice (Winkler 1977).
11. The male tester claps his hands loudly in front of the dog.
12. The male tester shouts and makes hitting and kicking movements in the direction of the dog just out of reach of the dog (Wright 1985).

The dog is moved to the corner of the testing room. Again, it is attached to a hook with a double leash by its owner. The owner is standing next to the dog during subtests 13-16.

13. Two people surround and approach the dog quickly, while staring at it.
14. The male tester threatens the owner by yelling and shouting at him/her and that tester pushes the owner with the artificial hand. The hand also moves in the direction of the dog several times (Beck *et al.* 1975; Seiferle and Leonhardt 1984).
15. Two people corner dog and owner with two female dogs on the leash.
16. A tester with a dominant dog on the leash approaches the dog, stopping at a distance of 0.5 m from the edge of the corner testing area. The gender of the stimulus dog is the same as the gender of the Golden Retriever (Goddard and Beilharz 1985).

The dog is transferred back to the central area.

17. A tester walks with the stimulus dog towards the owner (who is sitting on the chair) and the owner is asked to pet the stimulus dog and not to pay attention to his/her own dog (Goddard and Beilharz 1985).
18. The dog is given its feeding bowl by its owner at a distance of 0.5 m from the same stimulus dog (Goddard and Beilharz 1985).
19. The owner gives the feeding bowl of the Golden Retriever to the stimulus dog (Goddard and Beilharz 1985).

The owner leaves the room again through door 3, so subtest 20 and 21 are performed in the absence of the owner.

20. A life-sized doll 65 cm tall is taken at walking speed towards the dog by a tester. When reaching the dog, the tester tries to touch the dog with the hand of the doll (Blackshaw 1988; van der Borg *et al.* 1991; Wright 1985).
21. A tester wearing a dog mask approaches the dog.

The owner takes a seat on the chair again.

22. A tester pets the dog with the artificial hand.

These subtests are similar to subtests 1, 5, 6, 9, 11, 16, 17, 18, 21, 23, 27, 28, 31, 33, 34, 35, 36, 37, 38, 24 and 12 in the aggression test of Netto and Planta, except for some small practical alterations. For the sake of clarity, descriptive keywords will be added to subtest numbers in the remaining of this section.

The following small deviations from the protocol were allowed: the owner was present instead of absent during one of the subtests; the owner was standing instead of sitting during one or two subtests; the owner petted the dog for a short time at the start of one of the subtests; a tester instead of the owner gave the feeding bowl to the dog; and some of the subtests accidentally lasted more than twenty seconds (or one minute for subtest 4 and 5).

## Ethological and statistical analysis

All tests were recorded on videotape and subsequently analysed using an ethogram (Tables 1 and 2). Scoring was only performed during the twenty seconds or one minute a subtest lasted, also when the subtest accidentally took more time. We scored how often the dog showed each behavioural element during this period (continuous sampling) and these frequencies were added for the subtests. Subtests 1, 2, 3, and 22 were not included in the sum because their standardisation was moderate and the behaviour of the dogs during these subtests was sometimes poorly visible on tape. Note that subtests 1, 2, 3, and 22 are not excluded from the general test results presented in the results paragraph. The result of this ethological analysis was a "behavioural profile"

**Table 1.** Ethogram of aggressive dog behaviour.

| | |
|---|---|
| **direct staring** | The dog is staring at the stimulus. Often the pupils are slightly widened and the dog freezes. |
| **raising the hackles** | Hairs on neck, back and hindquarters rise. |
| **stiff posture** | Muscles in the body are tense; the dog looks stiff and does not move. |
| **barking** | Short barking sound. |
| **growl-barking** | Combination of growl and bark. |
| **growling** | Low buzzing sound. |
| **baring the teeth** | The dog pulls up its upper lip, so that its teeth are visible. |
| **pulling up the lip** | Lips are pulled up slightly, but teeth are not visible. |
| **snapping** | A snapping movement (mouth opens and closes, possibly accompanied by showing the teeth and/or growling and/or barking) associated with a short lunge forward (not maximally) or a quick head movement. |
| **attacking** | The dog quickly moves forward maximally and makes snapping movements or actually bites (this may be impossible because of the subtest safety design), possibly accompanied by showing the teeth and/or growling and/or barking. |

**Table 2.** Ethogram of fearful dog behaviour.

| | |
|---|---|
| **trembling** | The dog is trembling all over its body. |
| **attempting to flee** | The dog tries to increase the distance to the stimulus by moving forward or backward until the leash is stretched maximally. |
| **shrinking back** | The dog shrinks backward or sideward, away from the stimulus, but it does not use the full length of the leash. |
| **seeking cover** | The dog tries to hide behind its boss or somebody or something else with respect to the stimulus. |
| **support seeking** | The dog approaches its owner, looks at its owner, and/or pushes itself against its owner, but it does not hide behind its owner. |
| **tongue flicking** [1] | The tongue shortly appears from the front of the beak. |
| **licking the beak** [1] | The tongue shortly appears from the front of the beak and licks the upper lip with a lateral movement. |
| **breaking eye contact** | The dog obviously looks away from the stimulus for at least 3 seconds. |
| **lifting front paw** | The dog lifts one front paw and keeps standing like this for a short time. |
| **smacking the lips** | The dog opens and closes its beak; this is not a biting attempt and there is no movement forwards. |
| **hunching** | Hunching for a short time. |
| **startling movement** | Short startling movement (no hunching) of the whole body. |
| **squeaking** | High squeaking sound. |

[1] This behaviour was not scored during subtests 7, 8, 18 and 19, because these subtests all involved food and the behaviour was therefore considered to have no fear motivation.

for each dog, consisting of the frequency of the behaviours listed in Tables 1 and 2 during subtest 4 up to 21. We used three methods for converting the behavioural profiles into a behavioural phenotype:

1. The “snap/attack score”, i.e. the total number of snaps and attacks recorded during subtests 4 up to 21. Two-tailed Mann Whitney U tests were used to determine whether these scores corresponded to the owner-acknowledged classification. We used the non-parametric Mann Whitney U test to avoid assumptions about the underlying distribution of the variables.
2. The “total aggression score”, i.e. the added frequency of snapping, attacking, and the threatening behaviours listed in Table 1. The scores were again compared to the owner-acknowledged classification using Mann Whitney U tests.
3. The complete behavioural profiles of the dogs, including both aggressive and fearful behaviours, were subjected to principal factor analysis (PFA). The aim of this analysis was to reduce the large number of behavioural elements to a small number of underlying variables (factors) based on patterns of correlations between the behavioural elements. We may for example expect to find a factor consisting of aggressive behaviours and another factor consisting of fearful behaviours. The behavioural elements “raising the hackles”, “trembling”, and “smacking the lips” were excluded from the PFA because they occurred only incidentally. Scores on the behavioural elements “tongue flicking” and “licking the beak” were added, as were those on “seeking cover” and “support seeking”. SPSS software was used for the PFA; factors with eigenvalues over 1 were extracted and the varimax method was used for rotation. Two-tailed Mann Whitney U tests were used to determine whether different owner-acknowledged classes had different median factor scores.

For the sake of clarity, the results of the personal interviews, the general test results and the results of the three methods of analysis will each immediately be discussed in the results paragraph.

## Ethical aspects

We obviously did not want our test to have adverse effects on the future behaviour of the dogs. Thus, the test was aborted when it was too stressful for a dog and the owner could also terminate the test at any time. Dogs that did not complete the test were not included in this section. Chances were high that context learning occurred because the majority of the test was performed inside the test room (i.e. the dogs would associate the unpleasant experiences

of the test with the test room and not with situations that they may encounter again in the future). The possibility of a future increase of aggressive behaviour as a result of "winning experiences" during the test was minimised by trying to make sure that neither the stimulus dogs, nor the testers would ever be "defeated" by the Golden Retriever.

## Results

### Personal interviews

Based on the personal interviews, 24 dogs were classified as "owner-acknowledged non-aggressive" and 59 dogs were classified as "owner-acknowledged aggressive". The information provided by the owners showed that the aggressive behaviour was not similar in all dogs. The owner-acknowledged aggressive group included dogs that had bitten their victims once or several times ("biters", n=44) and dogs that had not bitten so far ("threateners", n=15). Some dogs were only aggressive to people (n=20); other dogs were exclusively aggressive to dogs (n=7); and others were aggressive to both people and conspecifics (n=32). Aggressive behaviour to people was sometimes restricted to family members, whereas other dogs were aggressive to strangers. There was also variation in the location where aggressive behaviour usually occurred (i.e. within the own territory of the dog or outside). Environmental influences are probably involved in this phenotypic heterogeneity, but genetic variation may also play a role. It is possible that a strong genetic basis is only present in a subgroup of the dogs, and, although unlikely within a single breed, it is also possible that different gene mutations are responsible for different phenotypes (i.e. genetic heterogeneity). The key to success in this research project may be to find a homogeneous subgroup of owner-acknowledged aggressive dogs in which aggression has the same genetic aetiology.

### General test results

All dogs performed the test according to the protocol. The majority (81 dogs) showed some of the aggressive behaviours listed in Table 1 during the test. We observed fearful behaviour (Table 2) in all dogs during several subtests. The two most intense aggressive behaviours, attacking and snapping, were observed in 29 Golden Retrievers (35%). This percentage is low compared to the 67% reported for the Netto and Planta dogs. The length of our test can probably

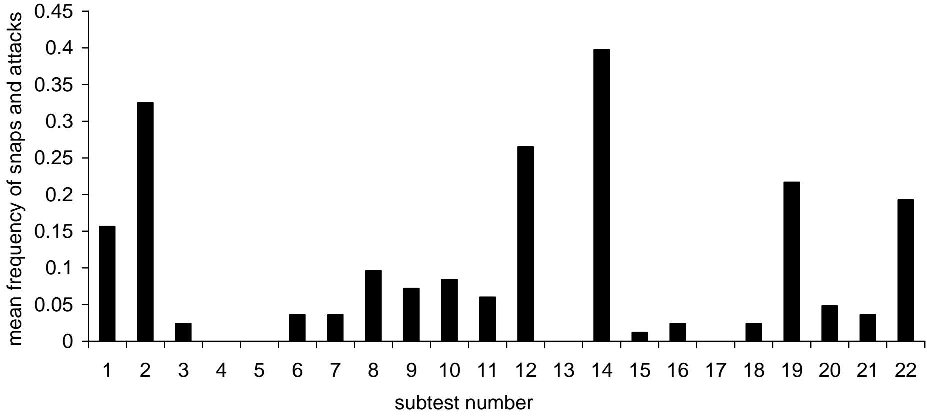
explain this; Netto and Planta showed that a test with more subtests elicits more aggressive behaviour. We abbreviated their test because Netto and Planta had a high number of "false positives" (owner-acknowledged non-aggressive dogs that attacked in the test). Another explanation is the breed of the subjects: all subjects in this study were Golden Retrievers. They are probably less aggressive and more easily impressed than the "potentially aggressive breeds" that Netto and Planta mainly tested.

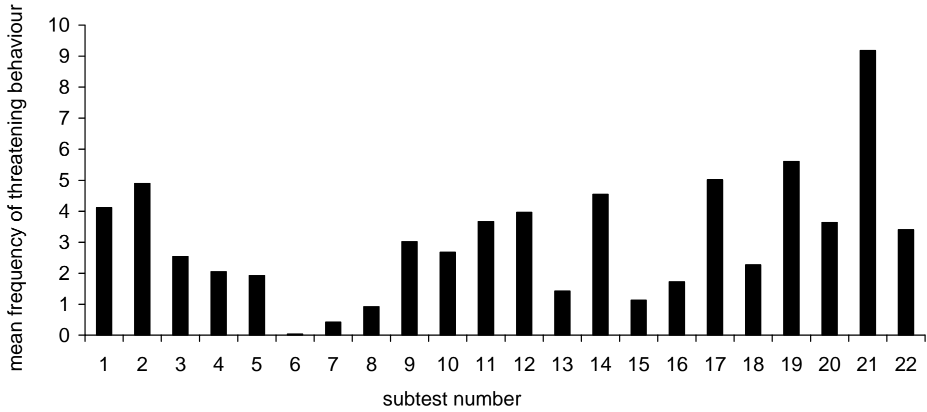
As expected from the Netto and Planta studies, the subtests varied in aggression-eliciting power. Ranking the subtests based on the mean number of snaps and attacks evoked in a dog gave the following order: 14-2-12-19-22-1-8-10-9-11-20-6/7/21-3/15/16/18-4/5/13/17, with subtest 14 (threatening the owner) eliciting the highest mean number of snaps and attacks (Figure 2a). Snapping and attacking never occurred during subtests 4 and 5 (both tug-of-war), 13 (cornering), or 17 (owner pets other dog). Netto and Planta expressed the aggression-eliciting power of subtests as the percentage of dogs that snapped or attacked during a subtest. This parameter gives the following descending order of subtests in the Golden Retrievers: 2/14/22-1/19-12-9-10-7/11/16-3/6/8/15/18/20/21-4/5/13/17. For the results presented by Netto and Planta, this order was: 19-18-14-17-15-16-12-1-3-11-13-6-20-10-9-7-8-22-2. These orders differ substantially. For instance, subtest 17 (owner pets other dog) evoked snap/attack behaviour in many dogs in the Netto and Planta test, whereas this behaviour was never recorded during our subtest 17.

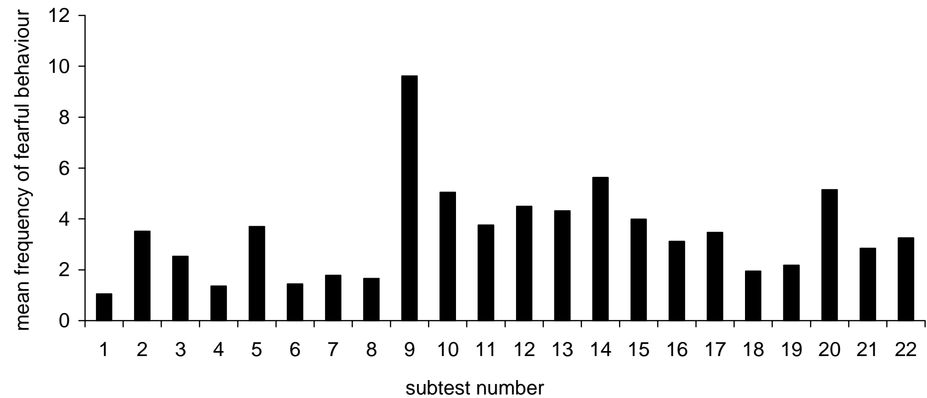
In all subtests threatening behaviour was elicited in several dogs. Ranking them based on the mean frequency of threatening behaviour resulted in the order: 21-19-17-2-14-1-12-11-20-22-9-10-3-18-4-5-16-13-15-8-7-6, with subtest 21 (dog mask) evoking the highest mean threatening frequency (Figure 2b). Aggressive behaviour during subtest 4 and 5 (both tug-of-war) was always barking or growling. This should probably be interpreted as play barking and/or play growling because the dogs showed no threatening postures during these subtests. Figure 2c shows that in all subtests fearful behaviour was elicited in some dogs and that subtest 9 (umbrella) evoked the highest mean fear frequency.

### Snap/attack scores as a measure of behavioural phenotype

A simple method of translating the test results into a phenotypic measure is to consider only the most intense aggressive behaviours: snapping and attacking. We calculated a "snap/attack score" for each dog, i.e. the total number of snaps and attacks recorded during subtests 4-21. For molecular genetic analysis, this score may either be used as a quantitative measure of


a. Snap/attack behaviour
mean frequency of snaps and attacks
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
subtest number


b. Threatening behaviour
mean frequency of threatening behaviour
0
1
2
3
4
5
6
7
8
9
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
subtest number


c. Fearful behaviour
mean frequency of fearful behaviour
0
2
4
6
8
10
12
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
subtest number

◄ **Figure 2.** Aggression- and fear-eliciting properties of the 22 subtests. For each subtest, the mean frequency of snapping and attacking behaviour of the dogs is depicted in figure a; the mean frequency of threatening behaviours (i.e. direct staring, raising the hackles, stiff posture, barking, growl-barking, growling, baring the teeth and/or pulling up the lip) is shown in figure b; and the mean frequency of fearful behaviour (i.e. trembling, attempt to flee, shrinking back, seeking cover/support, tongue flicking, licking the beak, breaking eye contact, lifting front paw, smacking the lips, hunching, startling movement, squeaking), is depicted in figure c. Numbers on the horizontal axes correspond to the subtests described in the Methods paragraph.

aggressiveness, or it may be used to classify dogs as either aggressive or non-aggressive. For instance, a dog could be classified as aggressive if it has a snap/attack score higher than zero. Sixty-two Golden Retrievers never snapped or attacked during subtest 4-21. Detailed information about the number of snaps and attacks of the animals is shown in Table 3.

Owner-acknowledged aggressive dogs had significantly higher snap/attack scores than dogs that were not aggressive according to their owner (p=0.018). This significance relies mainly on the test results of the 24 owner-acknowledged non-aggressive dogs: only two of them snapped or attacked in the test. There was more discrepancy in the scores of owner-acknowledged aggressive dogs: 40 of them (68%) did not attack or snap. Several explanations can be given for this discrepancy:

1. The test does not include all possible aggression-eliciting stimuli and it is mild because of its limited length. Exclusion of subtests 1, 2, 3, and 22 from the snap/attack scores lowered them because subtests 1, 2, and 22 evoked a lot of snapping and attacking (Figure 2a). Only 54% of owner-acknowledged aggressive dogs had a snap/attack

**Table 3.** Phenotypes expressed as snap/ attack scores for the two owner-acknow-ledged classes of Golden Retrievers. Subtests 1, 2, 3 and 22 were not included in the frequencies.

| Number of attacks or snaps recorded during subtest 4 up to 21 | Number of owner-acknow-ledged non-aggressive dogs | Number of owner-acknow-ledged aggressive dogs |
|---|---|---|
| 0 | 22 | 40 |
| 1 | 1 | 1 |
| 2 | 0 | 7 |
| 3 | 1 | 1 |
| 4 | 0 | 2 |
| 5 | 0 | 1 |
| 6 | 0 | 2 |
| 9 | 0 | 3 |
| 12 | 0 | 1 |
| 31 | 0 | 1 |

score of zero (compared to 68%) if subtests 1, 2, 3, and 22 were included in the scores. We conclude that the limited number of stimulus situations in the test is one of the causes of the disagreement between snap/attack scores and the impression of the dog owner.

2. The impression of the owner may be unreliable because owners may misinterpret the behaviour of their dog.

The information provided by the owners revealed that owner-acknowledged aggressive dogs form a heterogeneous group with respect to several characteristics. This provides additional explanations for the discrepancy:

3. The target species varied (people, dogs, or both). When these three subgroups were analysed individually, only dogs with a history of aggressive behaviour towards both humans and conspecifics had significantly higher snap/attack scores than owner-acknowledged non-aggressive dogs ($p=0.009$). Dogs that were reported to be aggressive towards humans only or towards dogs only were not significantly more likely to attack or snap during the test than owner-acknowledged non-aggressive dogs ($p=0.116$ and $p=0.444$, respectively). This will be discussed further in the paragraph dealing with total aggression scores.
4. Dogs that only bit familiar people in the past are not expected to bite the testers. Therefore, we used a $\chi^2$ test to compare the familiarity of the victims (family members or strangers) between owner-acknowledged aggressive dogs that did not snap or attack during the test and owner-acknowledged aggressive dogs that did. There was no significant difference between the two groups ($p=0.78$).
5. Dogs that are only aggressive in their own territory are not expected to show aggression in the test. We compared the location where the dogs had usually behaved aggressively (within their own territory, outside, or both) between owner-acknowledged aggressive dogs that did not snap or attack during the test and owner-acknowledged aggressive dogs that did. There was no significant difference between these groups ($p=0.73$).
6. We included threateners (i.e. dogs that had never bitten people or another dog) in the owner-acknowledged aggressive group. This might partially explain the discrepancy because the snap/attack score includes only biting behaviour. We repeated the analysis with biters only ($n=44$). Sixty-six percent of the biters did not attack or snap in the test, so excluding the threateners would slightly improve the agreement between the impression of the owner and the test results.

Taking into account explanation 1, 3, and 6, we compared snap/attack scores for the complete test (including subtest 1, 2, 3, and 22) of owner-acknowledged non-aggressive dogs ($n=24$) to those of dogs with a biting history towards both

humans and conspecifics (n=26). The aggressive dogs had significantly higher snap/attack scores, with a p-value of 0.00036. Only 42% of them did not snap or attack during the test. In conclusion, the snap/attack score seems to be a reasonable measure of aggressiveness for dogs with a biting history towards both humans and conspecifics. However, the overall usefulness of the score is questionable because of the low agreement with the impression of the owner in the total study group.

### Total aggression scores as a measure of behavioural phenotype

An alternative to the snap/attack method is using the "total aggression score" (i.e. the total frequency of the aggressive behaviours listed in Table 1 during subtest 4-21) as a behavioural phenotype. Like snap/attack scores, total aggression scores can be treated both as a quantitative measure of aggressiveness and as a basis for creating phenotypic classes (e.g. "aggressive" or "non-aggressive"). Total aggression scores varied from 0 to 362 in the Golden Retrievers (Table 4).

Owner-acknowledged non-aggressive dogs had significantly lower total aggression scores than owner-acknowledged aggressive dogs (p=0.008). The agreement between total aggression scores and owner-acknowledged classes was thus more significant than was the case for the snap/attack scores. However, some owner-acknowledged non-aggressive dogs had very high total aggression scores. Conversely, several owner-acknowledged aggressive dogs had very low scores (Table 4). Some of

**Table 4.** Phenotypes expressed as total aggression scores for the two owner-acknowledged classes of Golden Retrievers. Scores were grouped in classes. Please note that the size of the last class (180-362) differs from the others. Subtests 1, 2, 3 and 22 were not included here.

| Number of aggressive behaviours recorded during subtest 4 up to 21 | Number of owner-acknow-ledged non-aggressive dogs | Number of owner-acknow-ledged aggressive dogs |
|---|---|---|
| 0 - 19 | 17 | 19 |
| 20-39 | 1 | 10 |
| 40-59 | 2 | 8 |
| 60-79 | 0 | 3 |
| 80-99 | 1 | 6 |
| 100-119 | 1 | 4 |
| 120-139 | 0 | 3 |
| 140-159 | 1 | 2 |
| 160-179 | 0 | 2 |
| 180-362 | 1 | 2 |

the explanations for this disagreement that were mentioned in the snap/attack paragraph are also valid here, i.e. the limited number of stimuli presented in the test, the short duration of the test, and the moderate reliability of the owners story. As was already mentioned, aggressive behaviour during the tug-of-war subtests 4 and 5 should probably be interpreted as play barking and/or play growling. We also calculated total aggression scores without these two subtests. These adjusted scores were again compared between owner-acknowledged aggressive and non-aggressive dogs, but the resulting p-values were not significantly different from the ones where subtests 4 and 5 were included.

In the previous paragraph, we demonstrated that there were substantial differences in the agreement between the impression of the owner and snap/attack scores between three subgroups of owner-acknowledged aggressive dogs (aggressive to people, to dogs, or to both people and dogs). This also applies to total aggression scores. Although dogs with a history of aggression towards only people had significantly higher total aggression scores than owner-acknowledged non-aggressive dogs ($p=0.025$), the agreement was better for dogs with a history of aggression towards both people and conspecifics ($p=0.010$). There was no significant difference between the total aggression scores of owner-acknowledged non-aggressive dogs and dogs with a history of aggression towards only dogs ($p=0.595$). The explanation for this is probably that both snap/attack and total aggression scores are a measure of overall aggressiveness. They are both the sum of frequencies observed during subtests where humans are the threatening stimuli ("people-subtests") and subtests where conspecifics are the threatening stimuli ("dog-subtests"). Although dogs with a history of aggression towards exclusively humans may show aggression during people-subtests, their total aggression score does not necessarily have to be high because this score also includes behaviour during dog-subtests. The reverse is true for the dogs with a history of aggression towards exclusively conspecifics, and it is even worse in their case because the number of dog-subtests is smaller than the number of people-subtests. Addition of data from various subtests therefore seems to be an inadequate method of phenotyping for these two groups of dogs. It is probably more effective to separately analyze the behaviour during classes of subtests with similar stimuli for these dogs.

In conclusion, total aggression scores are more useful than snap/attack scores, because the former agree better with the impression of the owner. The higher variation in total aggression scores suggests that it is a more realistic measure of aggression than the snap/attack score.

**Principal factor analysis of aggressive and fearful behaviour**

In the third method of converting the behavioural profiles into phenotypes, we applied principal factor analysis (PFA) to the behavioural profiles of the dogs. PFA is aimed at identifying underlying variables (factors) that explain the pattern of correlations between behavioural elements. The factor scores on the factors of the PFA solution can be used to classify the dogs. As was the case for both snap/attack and total aggression scores, these factor scores can be treated both as a quantitative measure of aggression and/or fear, and as a basis for a qualitative classification of the dogs. Six factors were extracted from the data. They explained 66% of the total variance between the dogs. Factor loadings were generally moderate to high and there was little cross loading between the factors. The rotated factor matrix is shown in Table 5. Behavioural elements that loaded on the first factor were mainly threatening. More severe aggressive behaviours like attacking and snapping had the highest loadings on the second factor. The other four factors all consisted of fearful behavioural elements, with an emphasis on active behaviours like "attempting to flee" in the third factor, on startling in the fourth factor, on support seeking in the fifth factor, and on uncertainty in the sixth factor.

We only expected factor scores to differ between owner-acknowledged groups for the first two factors because the owner-acknowledged classification is based on aggressive behaviour and not on fearful behaviour. As expected, owner-acknowledged aggressive dogs had significantly higher factor scores on factor 1 ("threatening") than owner-acknowledged non-aggressive dogs (p=0.040). The difference between factor scores on factor 2 did not reach significance (p=0.065). Factor scores on the other four factors did not differ significantly between these owner-acknowledged groups, as we expected (p=0.34, p=0.26, p=0.25 and p=0.85 for respectively factors 3, 4, 5, and 6). Interestingly, factor scores on factor 1 did not differ significantly between owner-acknowledged non-aggressive dogs and the subgroups of owner-acknowledged aggressive dogs (aggressive to only people, aggressive to only dogs, or aggressive to both, p=0.195, p= 0.053, and p=0.139, respectively).

The results of this PFA have to be treated with caution. A drawback of PFA is that it is often hard to test the reliability of the solution (Tabachnick and Fidell 2001). Splitting the group of dogs in two random halves and then repeating the PFA is an appropriate method to test the stability of the solution. However, in this study the sample size in such split groups is too small for a reliable PFA because correlation coefficients tend to be less reliable when estimated from small samples (Tabachnick and Fidell 2001). It would be interesting to repeat the analysis with a higher number of dogs in the future.

**Table 5.** Rotated factor matrix resulting from principal factor analysis of the behavioural profiles of the dogs. The names of the factors are based on the behavioural elements that contributed most to them. Numbers in brackets represent the percentage of variance explained by the factors. Factor loadings between - 0.3 and 0.3 are not presented in the table.

| | Factor | | | | | |
|---|---|---|---|---|---|---|
| | **1: threate-ning (16.6%)** | **2: attacking (15.5%)** | **3: active fear (10.7%)** | **4: startling (7.9%)** | **5: support seeking (7.5%)** | **6: uncer-tainty (6.8%)** |
| **stiff posture** | 0.80 | | | | | |
| **direct staring** | 0.88 | | | | | |
| **growling** | 0.72 | | | | | |
| **pulling up the lip** | 0.74 | | | | | |
| **attacking** | | 0.88 | | | | |
| **baring the teeth** | | 0.94 | | | | |
| **barking** | | 0.75 | | | | -0.34 |
| **snapping** | 0.53 | 0.56 | | | | |
| **attempting to flee** | | | 0.83 | | | |
| **shrinking back** | | | 0.55 | 0.31 | | -0.42 |
| **seeking cover/ support** | | | 0.49 | | 0.60 | |
| **hunching** | | | 0.67 | | | |
| **startling movement** | | | | 0.68 | | |
| **lifting front paw** | | | | 0.61 | | |
| **growl-barking** | | | | -0.51 | | |
| **squeaking** | | | | | 0.73 | |
| **tongue flicking/ licking the beak** | | | | | | 0.77 |
| **breaking eye contact *** | | | | | | |

* This item had factor loadings between –0.3 and 0.3 on several factors and was therefore not classified in any of the factors.

Until then, we must regard the use of the factor scores as a promising but not completely reliable method of phenotyping the dogs.

We also performed a principal factor analysis on aggressive behaviours, fearful behaviours and postures. Postures are important in communication between dogs. The posture of a dog while behaving aggressively is an important indication of the motivation for the behaviour. The position of the ears, head and tail of the dog were used to determine the posture. However, this did not improve the solution of the PFA (results not shown).

# Discussion

## Standardisation

It is important to standardise test conditions (van der Staay and Steckler 2002), but it is hard to standardise behavioural tests like the one described here. The first three subtests were performed outside, so environmental variation was unavoidable (e.g. weather conditions, sounds, etc.). We therefore excluded these subtests from the analyses, but the dogs were nevertheless presented with slightly different situations prior to subtest 4 (tug-of-war). This may have caused variation in their test performance. There was also variation in the environment of the test room, e.g. air temperature and sounds coming from outside. In addition, the owners introduced variation when they did not always comply with our protocol (for instance standing instead of sitting). Testers accidentally varied the precise execution of the subtests. Scoring is also a source of considerable variation.

Two sources of variation could be prevented in the future. First, we used testers that were aware of the history of the dogs. They may have approached aggressive dogs differently than non-aggressive dogs. It is preferable to use ignorant testers in the future. Second, not all subtests were consistently performed by testers of the same gender. Subtests 9 (umbrella), 11 (clapping), 12 (hitting), and 14 (threatening the owner) were always performed by the male tester and subtest 10 (strange woman) was always performed by the female tester. However, subtest 3 (dominant dog behind fence), 5 (tug-of-war), 7 (pulling away feeding bowl), 16 (dominant dog), 20 (doll), 21 (dog mask) and 22 (petting) were performed by the male tester for some dogs, and by the female for other dogs. Although the tester is not the threatening stimulus in these subtests, it is better to standardise the gender of the tester because dogs respond differently to males than to females (Lore and Eisenberg 1986; Wells and Hepper 1999)

## Evaluation of the approach of addition of the subtests

The three methods of analysis presented in this section were all applied on a dataset where frequencies were added for various subtests. We have in fact added scores on various subtypes of aggression because classifications of aggressive behaviour in animals are often based on the nature of stimuli eliciting the behaviour (see section 1.3). This approach is based on the hypothesis that the Golden Retrievers have a genetically based lowered threshold for aggressive behaviour under various circumstances. This might for

instance be a manifestation of a lack of impulse control. Impulsivity has been studied in various animals and it is believed to be under control of the serotonergic system (Coccaro *et al.*, 1997; Feldman *et al.*, 1997; Peremans *et al.*, 2003; Soubrié and Bizot, 1990). It is possible that the aggressive behaviour in the Golden Retrievers is caused by a mutation in one of the genes of the serotonergic system.

Only one tool is available for evaluating the approach of addition of the subtests: a comparison with the impression of the owner. This owner provided information will not always be accurate, so we expected some discrepancy between the owner-acknowledged classification and test results. In addition, we used Mann Whitney U tests for all comparisons and these tests are normally used to compare two independent groups. The owner-acknowledged aggressive and non-aggressive group are not independent: most of the non-aggressive dogs are siblings of a number of aggressive dogs. Siblings have experienced a common early environment and they are genetically more similar than unrelated dogs. Therefore, they are expected to behave more similarly than unrelated animals and this will make it more difficult to find a significant difference in test results of the two groups. Keeping this in mind, the approach of addition of the subtests has given good results: both snap/attack scores, total aggression scores, and factor scores on the first aggressive factor in the PFA solution were significantly higher in owner-acknowledged aggressive dogs. However, the personal interview revealed that the group of owner-acknowledged aggressive dogs was heterogeneous. The agreement between test results and the impression of the owner was highest for the dogs with a history of aggression towards both people and conspecifics. This implicates that the approach of addition subtests is most adequate for this subgroup of owner-acknowledged aggressive dogs and maybe only these dogs have a lowered overall aggression threshold. It is an attractive idea is to use both test results and information provided by the owners for selecting a small homogeneous group of aggressive dogs for genetic analysis. For example, we could initially pick out the dogs that are aggressive to both people and conspecifics according to their owner and consequently further reduce the study group to dogs that had high total aggression scores in the test.

It will be very interesting to see the results of the second approach, where classes of subtests with similar stimulus situations will be analysed separately. We will show some results of this approach in section 2.2 of this thesis. The hypothesis underlying this approach is that different genetic mechanisms control different subtypes of aggressive behaviour. Naumenko *et al.* (1989) presented evidence for this hypothesis when they discovered that selection of Norway rats for reduced fear-induced aggression towards people

resulted in a decrease in irritable aggression, but no change in intermale and predatory aggression. Not much is known about the genetic basis of subtypes of canine aggression. It is claimed that fighting dogs have been selected exclusively for aggression towards dogs (Lockwood and Rindy, 1987), but there is no scientific evidence that this selection has not simultaneously increased aggressive behaviour towards people.

### One-zero sampling

The results presented in this section were based on a continuous sampling method. We also performed the calculations using one-zero sampling (i.e. scoring whether the dog shows the behaviour at least once or not at all during a subtest). Here, the agreement between the impression of the owner and snap/attack or total aggression scores was either identical or slightly lower than for continuous sampling (data not shown). The principal factor analysis did not give clear results (data not shown).

### Conclusion

The total aggression scores method was the best of the three methods that we presented in this section because it showed the best agreement with the impression of the owner and the highest variation in the study group. The results of the principal factor analysis were also promising. Addition of the behavioural frequencies observed during various subtests worked best for dogs that were owner-acknowledged aggressive to both people and dogs. This suggests that only these dogs may have a lowered “overall aggression threshold”. Analysis of classes of subtests will increase the insight into the aetiology of the aggressive behaviour. It remains to be seen whether the level of standardisation of the test meets the high requirements of molecular genetic studies of complex traits. The results from genetic studies in the Golden Retrievers will reveal this in the future.

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# 2.2

# Phenotyping of aggressive behaviour in Golden Retrievers with a questionnaire

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This section has been submitted for publication.

## Abstract

Reliable and valid phenotyping is crucial for our study of genetic factors underlying aggression in Golden Retriever dogs. A mail questionnaire based on the Canine behavioural assessment and research questionnaire (CBARQ; *Hsu and Serpell, 2003, JAVMA 223(9):1293-1300*) was used to assess behavioural phenotypes. Owners of 228 Golden Retrievers completed the questionnaire. These dogs had been referred to our clinic for aggression problems several years earlier or they were related to aggressive dogs. In this paper, three sets of results are presented, which indicate that behaviour scores from the CBARQ can be applied to genetic studies. First, factor analysis demonstrated that CBARQ items can be grouped into ten behavioural traits, including three types of aggression: stranger-directed aggression, owner-directed aggression, and dog-directed aggression. The results were remarkably similar to those reported by Hsu and Serpell. The aggression scores showed considerable variation in our dog families, which is a prerequisite for genetic studies. Second, retrospective questions enabled us to study changes in the aggressive behaviour of the dogs in the course of time. After an average time interval of 4.3 years, over 50% of the dogs had become less aggressive. Third, we analysed data obtained with an aggression test of 83 dogs. Two out of the three CBARQ aggression factors were also found in the aggression test data.

## Introduction

Aggressive behaviour is the most common behavioural problem in dogs, resulting in bite injuries of epidemic proportions (Beaver 1994; Lockwood 1995; Mikkelsen and Lund 2000; Mugford 1984). The nature and relative importance of genetic and environmental influences on canine aggression and their interaction are poorly understood. To approach these questions, we have embarked on a study of genetic factors underlying aggressive behaviour in dogs. Our studies focus on a single dog breed: the Golden Retriever. A friendly personality is characteristic of this breed. However, Golden Retrievers were the most frequently encountered purebred dogs in a group of Dutch dogs diagnosed with fear-motivated aggression (Galac and Knol 1997). This aggressive behaviour seemed to occur more often in certain family clusters than in others. It was thus suggested that genetic factors play an important role (Knol *et al.* 1997). Following these initial observations, we started building a database of aggressive Golden Retrievers and their relatives in 1997.

Purebred dogs provide a promising tool for studies of the genetic basis of behavioural traits. Breed barriers have led to strong genetic isolation of dog breeds, resulting in intrabreed genetic uniformity (Parker *et al.* 2004). Members of a dog breed display a morphological and behavioural uniformity, which sets them apart from other breeds. Dog behaviour could be a valid model for human behaviour because both dogs and humans show within group competition as well as cooperation (Overall 2000). With the completion of the dog genome project, the tools necessary for gene mapping studies in dogs have become readily available (Lindblad-Toh *et al.* 2005). The success of such studies depends on the availability of a reliable and valid method for phenotyping.

We previously used aggression tests for phenotyping (section 2.1). Although such tests are more objective than owner-derived information, their disadvantage is that it is difficult to evoke problem behaviour in a clinical setting. Indeed, we found that many Golden Retrievers that were aggressive according to their owner showed little or no aggression in the test (section 2.1). Questionnaires are regularly used in human behavioural genetics (e.g. Bartels *et al.* 2003). A reliable, validated questionnaire to assess canine behavioural traits was presented by Hsu and Serpell in 2003. Their questionnaire, now named the Canine behavioural assessment and research questionnaire (CBARQ), contains 76 items regarding aggression, fear and anxiety, trainability, excitability, separation-related behaviour, attachment, attention-seeking, and chasing. By means of factor analysis, Hsu and Serpell demonstrated that the CBARQ items could be grouped into several behavioural traits, including at least three types

of aggression: stranger-directed aggression, owner-directed aggression, and dog-directed aggression.

Hsu and Serpell (2003) evaluated the reliability of the CBARQ in a group of dogs of various breeds by calculating Cronbach's α, a measure of internal consistency. The reliability was acceptable for all but one of their factors. Svartberg (2005) studied the internal consistency reliability of CBARQ scores in a population of Swedish dogs of various breeds. He found Cronbach's alpha values roughly in line, though somewhat lower, with the values obtained by Hsu and Serpell. Other aspects of reliability, such as test-retest and inter-rater reliability were not reported. The factor structure of Hsu and Serpell (2003) seems to be stable across different populations of dogs because two other studies found similar factors (Goodloe and Borchelt 1998; Serpell and Hsu 2001). In an earlier study, Serpell and Hsu (2001) used a 40-item questionnaire for evaluating guide dog behaviour. Several items in this questionnaire were similar to CBARQ items. They performed factor analyses in three breed groups (Labrador Retriever, German Shepherd dog, and Golden Retriever) and found factors similar to those in the 2003 study. This suggests that the factor structure is also stable within dog breeds. Hsu and Serpell (2003) studied the validity of seven CBARQ scores by comparing them with clinical diagnoses from behavioural practitioners. Dogs assigned to particular diagnostic categories had significantly higher scores for corresponding CBARQ traits than those assigned to unrelated diagnostic categories. Svartberg (2005) used the CBARQ to validate personality traits derived from the Swedish dog mentality assessment. He demonstrated that the CBARQ factors "non-social fear" and "stranger-directed fear" correlated significantly with corresponding trait scores from the behavioural test, while there were no significant correlations between "chasing" and "stranger-directed aggression" and their corresponding trait scores.

The results of these studies suggest that the CBARQ is a reliable and valid method for evaluating canine behavioural traits. However, no studies have been devoted to the variation of CBARQ scores within dog families. We made a Dutch translation of the CBARQ and sent it to owners of 238 Golden Retrievers that had earlier participated in our project. In this section, three analyses will be presented. First, we evaluate the usefulness of the CBARQ as a method for phenotyping. Phenotypic scores derived from the CBARQ should meet at least three requirements in order to be useful for future gene mapping studies. First, they should be reliable, i.e. the results should be consistent. Second, they should lead to valid conclusions, i.e. they should be a good approximation of what they purport to measure (aggression in this case). Third, they should display variation within families of dogs. The second topic

that will be addressed here is the design and analysis of a shortened version of the CBARQ tailored for retrospective data on deceased dogs and for investigating behavioural changes over time. We made this shortened version because there was a gap of on average 4.3 years between the time that dogs participated in our project for the first time and the time the CBARQ was to be filled out. The behaviour of the dogs might have changed in the meantime and we expected a number of dogs to be euthanatized because of aggressive behaviour. Third, results will be presented of a further analysis of the aggression test data. In section 2.1, we analysed the behaviour of 83 Golden Retrievers in the aggression test. Behavioural scores were then obtained by summing frequencies of aggressive behavioural elements that were displayed in the subtests. Here, we investigate whether the subtests can be grouped into classes corresponding to CBARQ aggression types. Behavioural scores were now obtained by summing frequencies of aggressive behavioural elements in a class of subtests and the scores were compared to shortened CBARQ scores.

## Methods

### Subjects

Questionnaires were sent to owners of two groups of Golden Retrievers. Group 1 consisted of 126 Golden Retrievers that participated in our project in the period between October 1997 and October 2003 (Figure 1). The majority had been subjected to the aggression test as described in section 2.1. In addition, characteristics of their aggressive behaviour had been assessed through a personal interview with the owner at the time of testing. Group 1 contained dogs that were still alive as well as dogs that had died. Group 2 consisted of 110 dogs that joined the project between October 2003 and February 2005. These dogs were not subjected to the aggression test and there was no personal interview with the owner. Both groups contained dogs that were referred to our clinic because of their aggressive behaviour (probands) and dogs that were recruited by us because they were related to an aggressive dog (relatives).

Owners of 228 Golden Retrievers (97%) returned a completed questionnaire and 172 of these dogs were purebred with a pedigree. One hundred and ten were probands and 118 were relatives. There were 135 males (63 castrated) and 93 females (49 castrated) in the group. The mean age of the dogs was 6.6 years (range 6 months - 14 years) at the time the questionnaire was filled out. Characteristics of the subjects are listed by group in Table 1.

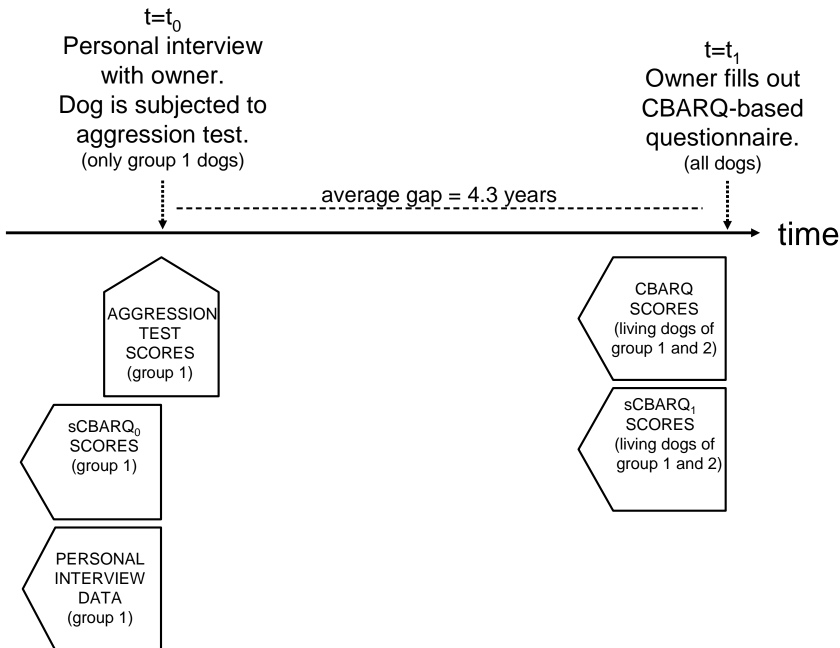



**Figure 1.** In this figure, five behavioural measures that were employed in our study are placed in a time frame. Owners of group 1 dogs participated in our project at two separate time points. There was an average gap of 4.3 years between these time points. During the first participation ($t=t_0$), the owners were personally interviewed and the majority of the dogs were subjected to an aggression test. During the second participation ($t=t_1$), the owner filled out a questionnaire based on the canine behavioural assessment and research questionnaire (CBARQ). This questionnaire also contained questions referring to the time of first participation (i.e. $sCBARQ_0$ questions). Owners of group 2 dogs were only asked to fill out the questionnaire based on the CBARQ. Block arrows below the time line represent the five behavioural measures. Positions of the arrows relative to the time line reflect the time frame they address. For instance, $sCBARQ_0$ questions address the behaviour of the dog in the months preceding the first participation.

## Design of the questionnaire

We developed three types of behavioural questions that were all based on the Canine behavioural assessment and research questionnaire (CBARQ): CBARQ items and two different types of shortened CBARQ questions. These questions will be described in more detail below. Table 2 provides an overview of the question types that were included in questionnaires for the different groups of dogs. Apart from the three types of behavioural questions, two additional types of questions were included in each questionnaire: questions about

**Table 1.** Characteristics of the subjects by group (st dev=standard deviation).

| | | **Sex** | | | | |
|---|---|---|---|---|---|---|
| **Group of subjects** | **Number of question-naires returned** | **Males (castrated)** | **Females (castrated)** | **Mean age (st dev)** | **Nr of pro-bands** | **Nr of pedigree dogs** |
| **Group 1** | | | | | | |
| living dogs | 84 | 55 (30) | 29 (21) | 7.5 (2.4) | 55 | 61 |
| deceased dogs | 36 | 26 (13) | 10 (4) | - | 28 | 19 |
| **Group 2** | | | | | | |
| living dogs | 108 | 54 (20) | 54 (24) | 5.9 (3.2) | 27 | 92 |
| **Total** | 228 | 135 (63) | 93 (49) | 6.6 (3.0) | 110 | 172 |

**Table 2.** Distribution of three types of behavioural questions over three versions of the questionnaire and number of questionnaires returned.

| | **Type of questions** | | | |
|---|---|---|---|---|
| **Version of questionnaire** | **$sCBARQ_0$ questions [1]** | **$sCBARQ_1$ questions [1]** | **CBARQ items [1]** | **Number of questionnaires returned** |
| **Group 1 [2]** | | | | |
| living dogs | + | + | + | 84 |
| deceased dogs | + | - | - | 36 |
| **Group 2 [3]** | | | | |
| living dogs | - | + | + | 108 |

[1] $sCBARQ_0$ questions addressed the behaviour of the dog in the months prior to its first participation in the project, while $sCBARQ_1$ questions and CBARQ items referred to the behaviour in the recent past. Plus signs mark included types of questions.
[2] Group 1 consists of dogs that had previously participated in our project.
[3] Group 2 consists of dogs that were recently recruited.

environmental factors (not discussed here) and questions regarding the age of the dog, its sex and reproductive status, and its weight. These questions were added at the end of the questionnaire.

## The canine behavioural assessment and research questionnaire

We translated the CBARQ into Dutch (Hsu and Serpell 2003; see Appendix I). All items in the questionnaire were worded to address the typical responses of the dog to specific situations and they were grouped in categories for simplicity (e.g. training and obedience, aggression, fear and anxiety). Owners were asked to score the behaviour of their dog with 5-point frequency scales (i.e. 0=never, 1=seldom, 2=sometimes, 3=usually, and 4=always) or 5-point qualitative rating scales (i.e. 0=no signs of the behaviour, 1 to 3=mild to moderate signs of the behaviour, and 4=severe signs of the behaviour). For these rating scales, a brief explanation was included describing the sorts of behavioural signs involved in the behaviour (e.g.: "Typical signs of moderate aggression in dogs include barking, growling, and baring teeth. More serious aggression generally involves snapping, lunging, biting, or attempting to bite."). The CBARQ is a modified version of the PennBARQ that was described in 2003; it contains several additional questions (marked in Appendix I) (J.A. Serpell, personal communication). We asked the owners to fill out the CBARQ only if their dog was still alive.

## Factor analysis and reliability of the factors

Principal factor analysis was performed on 71 CBARQ items with response rates of at least 92%. A total number of 184 dogs was included in the analysis; eight dogs with more than four missing values were excluded. Missing values were replaced with the mean. We used the scree test to determine the number of factors to be extracted. Subsequently, the varimax method was used to rotate the factor solution. A second factor analysis with the same procedure was performed on the items that were included in the most relevant factors of the first factor solution.

Cronbach's alpha was calculated in order to study the internal consistency reliability of the factors. Items were assigned to the factor on which they had the highest loading. Dogs with missing values in a factor were excluded from the calculation for that particular factor. Factors with a Cronbach's alpha value of 0.70 or higher were considered to be reliable.

## Calculation of behavioural scores from the CBARQ

We calculated behavioural scores as the mean of the CBARQ items that were grouped in a factor (e.g. stranger-directed aggression score = score for item 10+11+12+15+16+18+20+21+22+28/10). We will refer to these behavioural scores as "CBARQ scores" in the remaining of this section. An additional score was calculated as the mean of items 32-35. These items were added to the PennBARQ by Hsu and Serpell to represent familiar dog aggression (FDA; J.A. Serpell, personal communication). They were excluded from the factor analysis because of the low response rate. Items 43, 50, and 51 were excluded from calculation of CBARQ scores and item 64 was included in the "attachment, attention-seeking, and excitability" score instead of the chasing score in order to make the results more comparable to the Hsu and Serpell study (see Results and discussion). Median CBARQ scores of probands and relatives were compared with Mann-Whitney U tests.

## Shortened CBARQ questions

We designed a shortened version of the CBARQ (sCBARQ), in which only one question addressed each Hsu and Serpell factor (Appendix II). Two types of sCBARQ questions were made that differed in the time frame addressed. The first type of sCBARQ questions addressed the behaviour of the dog in the months prior to its first participation in the project (Figure 1). These will be referred to as "$sCBARQ_0$ questions". The second type of sCBARQ questions addressed the behaviour of the dog in the recent past. These will be referred to as "$sCBARQ_1$ questions". Table 2 provides an overview of the question types that were included in questionnaires for the different groups of dogs.

## Shortened CBARQ scores: comparison with CBARQ scores and reliability

All questionnaires about living dogs (192 dogs) contained both $sCBARQ_1$ questions and CBARQ items (Table 2). We used these questionnaires for calculations of linear regression of CBARQ scores on $sCBARQ_1$ scores. We compared linear, quadratic and cubic models. Linear models provided the best description of the relationship between the scores in all traits. For subsequent analyses, both $sCBARQ_0$ and $sCBARQ_1$ scores were converted using the regression formulas to make them more comparable to CBARQ scores.

The reliability of $sCBARQ_0$ scores was examined in group 1 dogs by comparison with data that were collected in the personal interview with the

owner at the time of testing. These personal interview data contained information about five behavioural traits: stranger-directed aggression, owner-directed aggression, dog-directed aggression, trainability, and non-social fear. We scored the information about aggressive behaviour and non-social fear on a binary scale, e.g. "Is your dog aggressive towards strangers or not?" Trainability was scored on a 3-point scale, i.e. good, average or bad. Mann-Whitney U tests or Kruskall-Wallis tests were used to compare the scores.

### Factor analysis of the aggression test

Details about the test procedure and the subjects can be found in section 2.1. Subtests 1, 2, 3, and 22 were not included in the analyses because their standardisation was moderate and the behaviour of the dogs was poorly visible on tape. Subtest 4 and 5 (both tug-of-war) were excluded because aggressive behaviour in these subtests should probably be interpreted as play.

Total frequencies of snapping, attacking and threatening behaviours (see Table 1 in section 2.1) in a subtest were summed to obtain "aggression scores per subtest". Factor analysis was performed on aggression scores in subtest 6-21. We extracted three factors (based on the scree test) and rotated the solution with the varimax method. We also performed factor analysis on fear scores per subtest (i.e. the sum of fearful behavioural elements observed in a subtest, see Table 2 in section 2.1). Here, we extracted factors with eigen-values over 1 and rotated the solution with the varimax method.

### Comparison of aggression test scores with $sCBARQ_0$ scores

Three aggression test scores were calculated. The "dog-directed aggression test score" was the sum of aggression scores in subtest 15-19. The "stranger-directed aggression test score" was the sum of aggression scores in subtest 9-12, 20, and 21. The "possessive aggression test score" was the sum of aggression scores in subtest 7, 8, and 12-14. Both aggression test scores and $sCBARQ_0$ scores were available for 70 Golden Retrievers. We compared these scores by calculating Spearman's rank correlation coefficients with adjustments for tied ranks.

### Statistical tests

SPSS software was used for all statistical analyses. The significance level $\alpha$ was set at 0.05. The sharper Bonferonni procedure of Hochberg (1988) was used to adjust for multiple testing within each group of tests.

## Results and discussion

### Response rates

Response rates were very high for the majority of CBARQ items (median 99.5%). Five items had response rates below 92%: four items about familiar dog aggression (response rate 55%) and one item about fear reactions when having claws clipped by a household member (78%). These low response rates were due to many owners that have only one dog and/or that do not clip the claws of their dog. Response rates for individual dogs ranged from 86% to 100% (median 100% when the five items with response rates below 92% were excluded). Response rates for shortened CBARQ questions were 100%.

### Factor analysis

Principal factor analysis was used to group 71 CBARQ items in ten factors that explained 58% of the total variance in item scores (Table 3). Items from the category "aggression" were grouped in four factors, which were labelled "stranger-directed aggression" (SDA), "owner-directed aggression" (ODA), "dog-directed aggression" (DDA) and "chasing" (CHASE). The CHASE factor also contained item 64 from "excitability" (excitement when the doorbell rings) and items from the category "miscellaneous" (dog chases cats, birds, or other animals). The majority of items from the category "fear and anxiety" were grouped in three factors: "stranger-directed fear" (SDF), "non-social fear" (NSF), and "dog-directed fear" (DDF). Items 43 (fear of veterinarian), 50 (fear when groomed or bathed), and 51 (fear when having feet towelled) loaded mainly on ODA, NSF, and SDF. Items from the categories "attachment and attention-seeking" and "excitability", with the exception of item 64, were grouped in one factor, which was labelled "attachment, attention-seeking, and excitability" (AAS&EX). The "separation-related behaviour" items formed one factor (SRB), but item 60 (destructive behaviour) and 61 (loss of appetite) loaded very low on this factor. Items from the category "training and obedience" were grouped in one factor (TRAIN). Some items had cross-loading on other factors (e.g. SDA and SDF). Communalities (i.e. the variance in an item accounted for by the ten factors) were moderately high for most items (see last column in Table 3; Tabachnick and Fidell 2001).

The factor solution presented here is very similar to the one obtained by Hsu and Serpell (2003). This stability of the factor structure is remarkable for three reasons. First, the subjects in our study were a selected group: all dogs

**Table 3.** Results of factor analysis on 71 CBARQ questionnaire items for 184 dogs.

| Hsu and [1] Serpell factor | CBARQ[1] item | SDA[2] (8.1%) | ODA[2] (7.2%) | AAS&EX[2] (7.0%) | NSF[2] (5.9%) | TRAIN[2] (5.7%) | SDF[2] (5.6%) | SRB[2] (5.3%) | DDA[2] (4.7%) | DDF[2] (4.5%) | CHASE[2] (4.3%) | Commu nality |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stranger-directed aggression | 10 | 0.76 | | | | | *0.32* | | | | | 0.73 |
| | 11 | 0.65 | | | | | *0.38* | | | | | 0.60 |
| | 12 | 0.48 | | | | | | | | | | 0.45 |
| | 15 | 0.66 | | | | | | | | | | 0.55 |
| | 16 | 0.75 | | | | | | | | | | 0.60 |
| | 18 | 0.59 | | | | | | | | | | 0.56 |
| | 20 | 0.68 | | | | | | | | | | 0.69 |
| | 21 | 0.68 | | | | | *0.46* | | | | | 0.73 |
| | 22 | 0.72 | | | | | | | | | | 0.61 |
| | 28 | 0.55 | | | | | *0.30* | | | | | 0.56 |
| Owner-directed aggression | 9 | | 0.56 | | | | | | | | | 0.59 |
| | 13 | | 0.70 | | | | | | | | | 0.64 |
| | 14 | | 0.63 | | | | | | | | | 0.47 |
| | 17 | | 0.73 | | | | | | | | | 0.70 |
| | 19 | | 0.71 | | | | | | | | | 0.70 |
| | 25 | | 0.67 | | | | | | | | | 0.52 |
| | 30 | | 0.62 | | | | | | | | | 0.53 |
| | 31 | | 0.74 | | | | | | | | | 0.70 |
| Stranger directed fear | 36 | | | | | | 0.64 | | | | | 0.66 |
| | 37 | | | | | | 0.66 | | | *0.31* | | 0.66 |
| | 39 | | | | | | 0.67 | | | | | 0.58 |
| | 40 | *0.35* | | | | | 0.74 | | | | | 0.77 |
| Trainability | 1 | | | | | 0.73 | | | | | | 0.58 |
| | 2 | | | | | 0.73 | | | | | | 0.57 |
| | 3 | | | | | 0.75 | | | | | | 0.62 |
| | 4 | | | | | 0.79 | | | | | | 0.64 |
| | 5 | | | | | 0.61 | | | | | | 0.43 |
| | 6 | | | | | 0.56 | | | | | | 0.42 |
| | 7 | | | | *-0.30* | 0.53 | | | | | | 0.50 |
| | 8 | | | | | 0.55 | | | | | | 0.39 |

**Table 3, continued.**

| Hsu and [1] Serpell factor | CBARQ[1] item | SDA[2] (8.1%) | ODA[2] (7.2%) | AAS&EX[2] (7.0%) | NSF[2] (5.9%) | TRAIN[2] (5.7%) | SDF[2] (5.6%) | SRB[2] (5.3%) | DDA[2] (4.7%) | DDF[2] (4.5%) | CHASE[2] (4.3%) | Communality |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Separation-related behaviours | 54 | | | | | | *0.31* | 0.62 | | | | 0.53 |
| | 55 | | | | | | | 0.65 | | | | 0.52 |
| | 56 | | | | | | | 0.62 | | | | 0.57 |
| | 57 | | | | | | | 0.71 | | | | 0.55 |
| | 58 | | | | | | | 0.63 | | | | 0.55 |
| | 59 | | | | | | | 0.82 | | | | 0.71 |
| | 60* | | | | | | | | | | | 0.38 |
| | 61 | | | | | | | 0.35 | | | | 0.26 |
| Excitability | 62 | | | 0.59 | | | | | | | | 0.57 |
| | 63 | | | 0.59 | *0.40* | | | | | | | 0.60 |
| | 64 | *0.33* | | | | | | | | | 0.43 | 0.53 |
| | 65 | | | 0.55 | | | | | | | | 0.44 |
| | 66 | | | 0.51 | | | | | | | | 0.43 |
| | 67 | | | 0.58 | | | | | | | | 0.57 |
| Attachment / attention seeking | 68 | | | 0.48 | | | | | | | | 0.36 |
| | 69 | | | 0.73 | | | | | | | | 0.64 |
| | 70 | | | 0.77 | | | | | | | | 0.64 |
| | 71 | | | 0.75 | | | | | | | | 0.63 |
| | 72 | | | 0.47 | | | | | | | | 0.47 |
| | 73 | | | 0.61 | | | | | | | | 0.50 |
| Nonsocial fear | 38 | | | | 0.72 | | | | | | | 0.65 |
| | 41 | | | | 0.56 | | *0.31* | | | | | 0.50 |
| | 42 | | | | 0.75 | | | | | | | 0.63 |
| | 44 | | | | 0.58 | | | | | | | 0.40 |
| | 47 | | | | 0.50 | | | | | | | 0.53 |
| | 48 | | | | 0.75 | | | | | | | 0.70 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Chasing | 27 | *0.39* | | | | | | | | | 0.41 | 0.49 |
| | 74 | | | | | | | | | | 0.84 | 0.75 |
| | 75 | | | | | | | | | | 0.74 | 0.62 |
| | 76 | | | | | | | | | | 0.79 | 0.69 |
| Dog-directed aggression and fear | 23 | | | | | | | | 0.81 | | | 0.78 |
| | 24 | | | | | | | | 0.79 | | | 0.74 |
| | 26 | | | | | | | | 0.74 | | | 0.71 |
| | 29 | | | | | | | | 0.71 | | | 0.59 |
| | 45 | | | | | | | | | 0.83 | | 0.77 |
| | 46 | | | | | | | | | 0.80 | | 0.70 |
| | 52 | | | | | | *0.32* | | | 0.65 | | 0.60 |
| | 53 | | | | | | | | | 0.78 | | 0.68 |
| Pain sensi-tivity | 43 | | *0.31* | | 0.45 | | | | | | | 0.52 |
| | 50 | | *0.34* | | | | 0.40 | *0.35* | | | | 0.55 |
| | 51 | | *0.52* | | | | 0.55 | | | | | 0.71 |
| Cronbach's α (nr of dogs) [3] | | 0.90 (180) | 0.87 (169) | 0.86 (185) | 0.82 (182) | 0.82 (184) | 0.83 (189) | 0.76 (189) | 0.88 (178) | 0.86 (176) | 0.78 (188) | |
| Cronbach's α excluding dogs with only zero's (nr of dogs) [3] | | 0.86 (98) | 0.80 (67) | 0.86 (185) | 0.78 (152) | 0.82 (184) | 0.74 (72) | 0.64 (66) | 0.85 (138) | 0.76 (84) | 0.72 (171) | |

[1] The first two columns show the Hsu and Serpell factor solution (2003).
[2] Column three through twelve depict our own factor solution. Numbers in brackets represent the percentage of variance explained by our factors. Loadings between –0.3 and 0.3 are not shown and cross-loadings are printed in italics. SDA=stranger-directed aggression; ODA=owner-directed aggression; AAS&EX=attachment, attention seeking and excitability; NSF=non-social fear; TRAIN=trainability; SDF=stranger-directed fear; SRB=separation-related behaviours; DDA=dog-directed aggression; DDF=dog-directed fear; CHASE=chasing.
[3] The two rows at the bottom of the table show Cronbach's alpha for our factors. Dogs with missing values on one or more items were excluded from the calculations; numbers in brackets represent the number of dogs included. Alpha was calculated both for the complete group of dogs (upper row) and for dogs with a higher score than zero on at least one of the items in the factor (lower row).
* Item 60 had factor loadings between –0.3 and 0.3 on several factors and was therefore not classified in any of the factors.

were Golden Retrievers that had aggression problems when they first participated in our project, or that were related to a dog with aggression problems. Second, we did not use the same set of items for the analysis: the CBARQ contains several new items that Hsu and Serpell did not include in their factor analysis. This is likely to affect the correlation matrix used for factor analysis. Third, the stability is remarkable because the number of dogs that was used in our factor analysis (184) is low compared to the number of items included (71). A variable-to-case ratio of 1:5 is often used as a lower boundary to create a stable correlation matrix. Taken together, these findings provide a firm support for the stability of the factor structure proposed by Hsu and Serpell across various populations of dogs.

We did observe some differences between our factor solution and the one presented by Hsu and Serpell (2003). First, the distinct factors DDA and DDF of our study were merged into a single "dog-directed aggression and fear" factor in the Hsu and Serpell solution. Second, the factor AAS&EX was split in a separate "attachment/attention-seeking behaviour" factor and an "excitability" factor by Hsu and Serpell. Item 64 (excitement when the doorbell rings) loaded on "excitability", instead of "chasing" in the Hsu and Serpell solution. We performed several exploratory factor analyses on our dataset by in- and excluding dogs and CBARQ items. The association between item 64 and CHASE was not stable. We decided to include the item in the AAS&EX factor in further analyses. Hsu and Serpell grouped items 43 (fear of veterinarian), 49 (fear when having claws clipped), and 50 (fear when groomed or bathed) in a factor "pain sensitivity". This factor had low internal consistency reliability (Cronbach's $\alpha$=0.60) in the study by Svartberg (2005). Associations between pain sensitivity items and our factors were not stable. We therefore decided to exclude item 43, 49, and 50 from further analyses.

A second factor analysis was performed on 36 items from the six aggression and fear factors of the initial solution (SDA, ODA, DDA, SDF, NSF, and DDF) in order to study the correlations between these items in more detail. The items were grouped in six factors that explained 63% of the variance in item scores (Table 4). The items of the factors ODA, NSF, DDA, and DDF behaved similarly as in the previous solution. SDA items 10, 11, and 21 (aggression when approached or petted by an unfamiliar adult or child) now loaded high on the SDF factor. These three items already had cross-loading on SDF in the previous solution. The remaining SDA items loaded on the main SDA factor, which seems to represent territorial aspects of SDA (aggression when strangers approach the dog while it is in the owner's car, when strangers approach the owner, and when mailmen, joggers or other strangers approach the home while the dog is in the yard).

**Table 4.** Result of factor analysis on 36 CBARQ aggression and fear items for 184 dogs.

| Factor[1] in first solution | CBARQ[1] item: | SDA-T[2] (13.1%) | ODA[2] (12.1%) | SDA&F[2] (10.9%) | NSF[2] (9.6%) | DDA[2] (9.1%) | DDF[2] (8.4%) | Com mu nality |
|---|---|---|---|---|---|---|---|---|
| Stranger-directed aggression | 10 | 0.67 | | *0.46* | | | | 0.72 |
| | 11 | *0.49* | | 0.57 | | | | 0.61 |
| | 12 | 0.48 | | | | *0.31* | | 0.42 |
| | 15 | 0.71 | | | | | | 0.56 |
| | 16 | 0.69 | | | | | | 0.60 |
| | 18 | 0.67 | | | | | | 0.56 |
| | 20 | 0.78 | | | | | | 0.73 |
| | 21 | *0.49* | | 0.65 | | | | 0.74 |
| | 22 | 0.78 | | | | | | 0.68 |
| | 28 | 0.66 | | | | | | 0.54 |
| Owner-directed aggression | 9 | | 0.57 | | | | | 0.47 |
| | 13 | | 0.77 | | | | | 0.66 |
| | 14 | | 0.60 | | | | | 0.39 |
| | 17 | | 0.81 | | | | | 0.78 |
| | 19 | | 0.78 | | | | | 0.76 |
| | 25 | | 0.64 | | | | | 0.48 |
| | 30 | | 0.59 | | | | | 0.42 |
| | 31 | | 0.78 | | | | | 0.68 |
| Stranger directed fear | 36 | | | 0.72 | | | | 0.65 |
| | 37 | | | 0.78 | | | | 0.72 |
| | 39 | | | 0.68 | | | | 0.57 |
| | 40 | | | 0.85 | | | | 0.83 |
| Nonsocial fear | 38 | | | | 0.79 | | | 0.70 |
| | 41 | | | | 0.62 | | | 0.51 |
| | 42 | | | | 0.81 | | | 0.71 |
| | 44 | | | | 0.62 | | | 0.42 |
| | 47 | | | | 0.54 | | *0.30* | 0.51 |
| | 48 | | | | 0.82 | | | 0.74 |
| Dog-directed aggress. | 23 | | | | | 0.87 | | 0.81 |
| | 24 | | | | | 0.85 | | 0.77 |
| | 26 | | | | | 0.78 | | 0.68 |
| | 29 | | | | | 0.71 | | 0.59 |
| Dog-directed fear | 45 | | | | | | 0.84 | 0.75 |
| | 46 | | | | | | 0.81 | 0.70 |
| | 52 | | | *0.34* | | | 0.67 | 0.60 |
| | 53 | | | | | | 0.81 | 0.70 |

[1] The first two columns show the factors that were found in the first analysis (Table 3).
[2] Column three through eight depict the new factor solution. Factor labels are presented above the columns and numbers in brackets represent the percentage of variance explained by the factors. Loadings between –0.3 and 0.3 are not shown and cross-loadings are printed in italics. SDA-T=territorial aspects of stranger-directed aggression; ODA=owner-directed aggression; SDA&F=stranger-directed aggression and fear; NSF=non-social fear; DDA=dog-directed aggression; DDF=dog-directed fear.

Hsu and Serpell (2003) did not mention correlations between SDA and SDF items, but in their earlier guide dog study, SDA and SDF items were highly correlated (Serpell and Hsu 2001). We can conclude that, at least in some dogs, aggression toward strangers and fear of strangers are associated. The suggestion that the aggressive behaviour towards strangers of some of our subjects is motivated by fear is supported by earlier reports that the familial aggression in Golden Retrievers is fear-motivated (Galac and Knol 1997; Knol *et al.* 1997). In the same line of reasoning, we interpret the correlations between the ODA factor and items 50 and 51 (fear when groomed, bathed, or having feet towelled) as a suggestion that the aggressive behaviour towards the owner of some of the subjects is also motivated by fear.

**Reliability of the factors**

Cronbach's alpha values ranged from 0.76 to 0.90 for our factors (Table 3), indicating that they are reliable. Alpha was 0.81 for items 32-35 (number of dogs = 102), suggesting that these items measure a single latent construct (familiar dog aggression). We also performed Cronbach's alpha calculations excluding dogs with a score of zero on all factor items because such dogs do not provide much information about the relationships between the items. Alpha was lower than for the complete group of dogs, but still higher than 0.7 in the majority of factors (Table 3). We therefore conclude that the factors are reliable in these dogs as well.

Although the above results provide firm evidence for the reliability of CBARQ factors, we would like to add one suggestion for future research. Aggression is evaluated on a 5-point qualitative rating scale in the CBARQ (i.e. 0=no aggression, 1 to 3=moderate aggression, and 4=serious aggression). Some Golden Retriever owners experienced difficulties answering these items because their dog would sometimes behave aggressively in a particular situation, while showing no signs of aggression at other times in the same situation. We expect such confusion of the owners to lead to decreased inter-rater reliability of the CBARQ scores. In the guide dog study of Serpell and Hsu, aggression items were scored on a frequency scale instead of a qualitative scale (e.g. "How often did your dog growl when approached by an unfamiliar adult in the recent past?"). This type of scale may be more appropriate for evaluating aggressive behaviour because it will introduce a higher inter-rater reliability. This should be investigated in the future.

## Variation in CBARQ scores

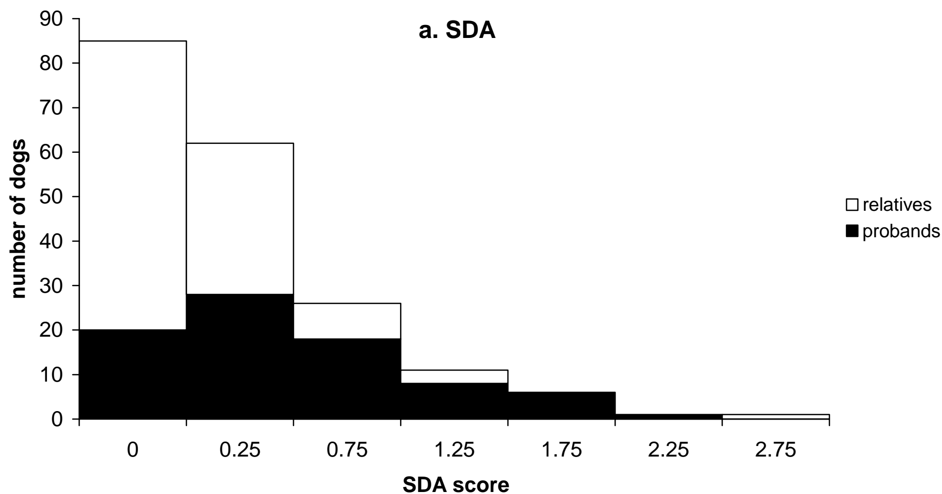
We calculated "CBARQ scores" as the mean of CBARQ items that were grouped in a factor of the solution from Table 3 (see methods). SDA scores of the 192 Golden Retrievers ranged from 0 to 2.8, with a mean of 0.34 (Table 5). ODA scores ranged from 0 to 2.9, with a mean of 0.32; and DDA scores ranged from 0 to 4, with a mean of 0.92. Dogs that were originally recruited as probands scored significantly higher than dogs that were recruited as relatives on all three types of aggression ($p<0.0001$ for SDA, ODA, and DDA; tested with Mann-Whitney U tests). Descriptive statistics of the other CBARQ scores can be found in Table 5.

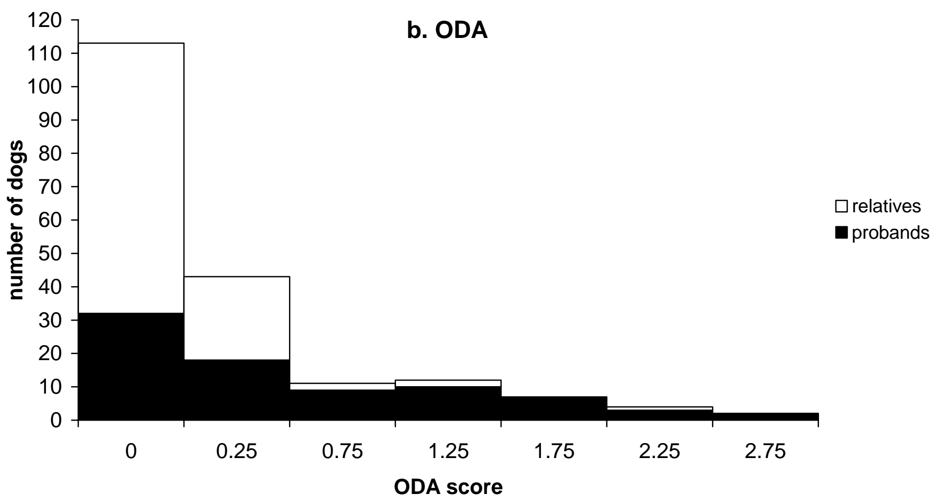
**Table 5.** Descriptive statistics of CBARQ scores in 192 Golden Retrievers.

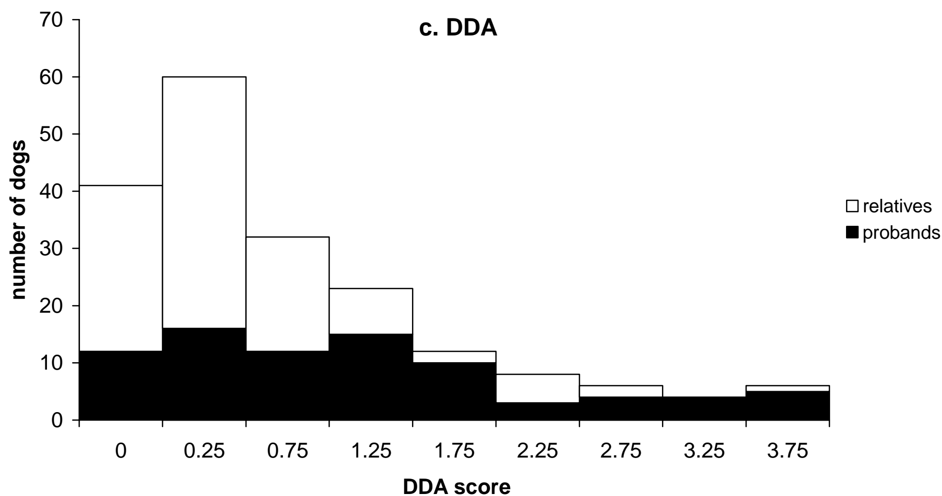
| | Complete group | | | | | Probands only | | |
|---|---|---|---|---|---|---|---|---|
| **CBARQ score[1]** | **Number of dogs** | **Mini-mum** | **Maxi-mum** | **Mean** | **St dev** | **Number of dogs** | **Mean** | **St dev** |
| **SDA** | 192 | 0 | 2.8 | 0.34 | 0.52 | 82 | 0.56 | 0.59 |
| **ODA** | 192 | 0 | 2.9 | 0.32 | 0.60 | 82 | 0.61 | 0.77 |
| **AAS&EX** | 192 | 0.080 | 3.4 | 1.6 | 0.76 | 82 | 1.9 | 0.80 |
| **NSF** | 192 | 0 | 3.7 | 0.83 | 0.81 | 82 | 1.2 | 0.95 |
| **TRAIN** | 192 | 0.50 | 4.0 | 2.8 | 0.69 | 82 | 2.7 | 0.70 |
| **SDF** | 192 | 0 | 4.0 | 0.23 | 0.60 | 82 | 0.35 | 0.68 |
| **SRB** | 189 | 0 | 1.6 | 0.16 | 0.32 | 81 | 0.24 | 0.41 |
| **DDA** | 192 | 0 | 4.0 | 0.92 | 1.0 | 82 | 1.3 | 1.2 |
| **DDF** | 192 | 0 | 3.3 | 0.49 | 0.71 | 82 | 0.52 | 0.74 |
| **CHASE** | 191 | 0 | 4.0 | 1.4 | 1.0 | 82 | 1.4 | 1.1 |
| **FDA** | 109 | 0 | 3.5 | 0.60 | 0.82 | 37 | 1.0 | 1.1 |

[1] SDA=stranger-directed aggression; ODA=owner-directed aggression; AAS&EX= attachment, attention seeking and excitability; NSF=non-social fear; TRAIN=trainability; SDF=stranger-directed fear; SRB=separation-related behaviours; DDA=dog-directed aggression; DDF=dog-directed fear; CHASE=chasing; FDA=familiar dog aggression.

The frequency distributions of SDA, ODA, and DDA scores were skewed to the right (Figure 2). Apparently, the majority of dogs had low aggression scores, in spite of the fact that almost half of them had been referred to our clinic for aggression problems several years before the questionnaire was filled out. Two explanations for these low aggression scores


a. SDA
number of dogs
0
10
20
30
40
50
60
70
80
90
0
0.25
0.75
1.25
1.75
2.25
2.75
SDA score
relatives
probands


b. ODA
number of dogs
0
10
20
30
40
50
60
70
80
90
100
110
120
0
0.25
0.75
1.25
1.75
2.25
2.75
ODA score
relatives
probands


c. DDA
number of dogs
0
10
20
30
40
50
60
70
0
0.25
0.75
1.25
1.75
2.25
2.75
3.25
3.75
DDA score
relatives
probands

◄ **Figure 2.** Frequency distributions of CBARQ aggression scores in 192 Golden Retrievers. The height of the bars represents the absolute number of dogs with a particular stranger-directed (a), owner-directed (b) or dog-directed (c) aggression score. Black parts of the bars represent probands and white parts represent relatives. Numbers underneath the bars represent class marks. Note that the size of the first class differs from the others: this class contains CBARQ scores of 0.

can be considered. First, there was an average gap of 4.3 years between the first participation and the time when the owner filled out the CBARQ. Many aggression problems that were present at the time of first participation have diminished over time. This is dealt with in detail later in this section. Another possible explanation for the low CBARQ scores is that the CBARQ contains a limited number of aggression-eliciting situations. It is possible that some other situations that provoke aggression in the Golden Retrievers are not present in the questionnaire. In order to investigate the plausibility of this explanation, we studied answers that owners gave to the open question "Are there any other situations in which your dog is sometimes aggressive?". No consistent pattern could be deduced from the answers of the Golden Retriever owners, indicating that the CBARQ encompasses the most important aggression-eliciting situations for our dogs. Low aggression scores can therefore not be explained by the absence of important aggression-eliciting situations in the CBARQ.

Phenotypic variation within families is a prerequisite for genetic studies. Figure 3 depicts CBARQ scores in a pedigree containing 42 Golden Retrievers that were included in the present study. There is considerable variation in aggression scores within this family. There is also a co-occurrence of different types of aggression in many dogs: of 22 dogs that had above-average scores on one type of aggression, 13 also scored above the mean for another type of aggression. It has been recognised by other authors that dogs regularly exhibit more than one type of aggression (Beaver 1993; Landsberg *et al.* 2004; Overall 1997). Note that there is no particular type of aggression that is clearly the most prevalent in this family. Both the co-occurrence of different types of aggression within single dogs and the presence of several types of aggression within this pedigree of closely related dogs may be due to genetic factors influencing all traits, to environmental factors influencing all traits, or to both.

### Shortened CBARQ scores: comparison with CBARQ scores and reliability

We designed a shortened version of the CBARQ for two purposes. First, we wanted to obtain behavioural scores for deceased dogs and we felt it was

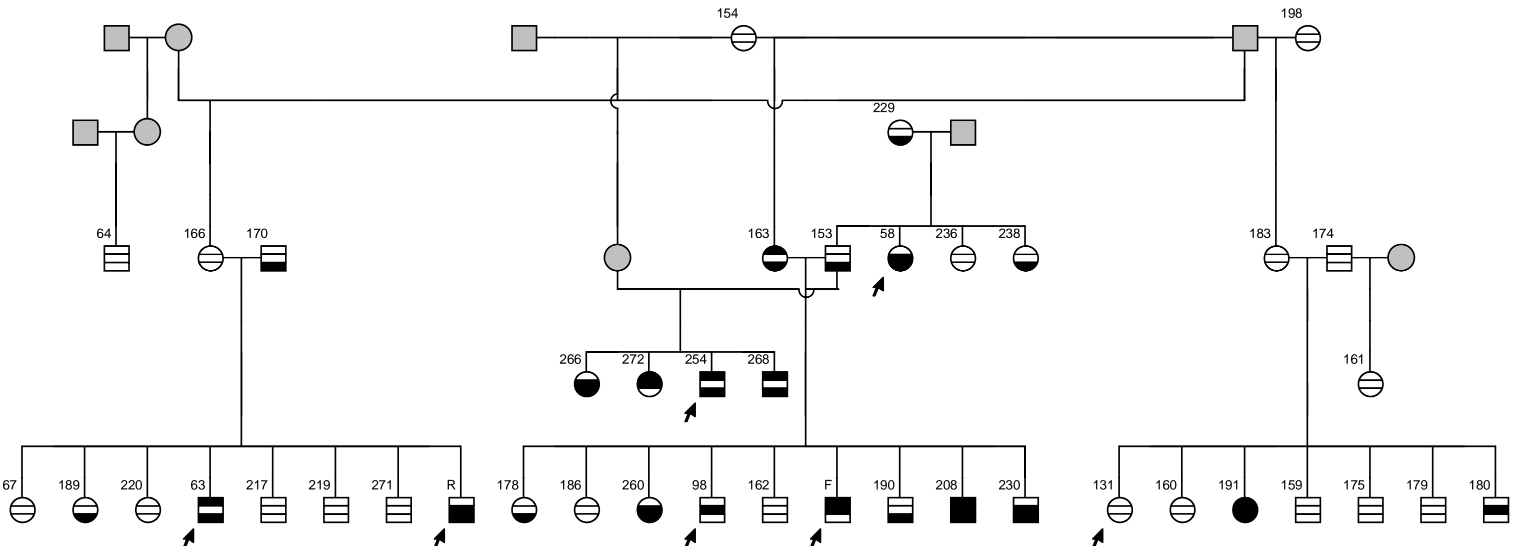



**Figure 3.** Pedigree containing 42 subjects of the present study. Squares represent males; circles represent females; and arrows mark probands. Numbers above the symbols are identification numbers from our database. Each symbol contains three behavioural phenotypes: the upper part of the symbol represents the stranger-directed aggression score (SDA); the central part represents the owner-directed aggression score (ODA); and the bottom part represents the dog-directed aggression score (DDA). Phenotypes are presented relative to mean of the complete group of 192 Golden Retrievers (0.34 for SDA, 0.32 for ODA, and 0.92 for DDA). A black shade is used if the dog has an aggression score above the mean of the complete group and a white shade is used for scores equal to or below the mean. Grey symbols represent ancestors from which we have no behavioural information. We have omitted all siblings from which behavioural information was not available. The phenotypes of dog R and 183 are based on shortened CBARQ questions.

inappropriate to send the long CBARQ to owners of deceased dogs. Second, we wished to investigate behavioural changes over time. Before we used sCBARQ questions to this end, we investigated the relationship between CBARQ scores and shortened CBARQ scores. We therefore performed linear regression of CBARQ scores on $sCBARQ_1$ scores. The coefficients are presented in Table 6. The coefficient of determination ($R^2$) ranged from 0.43 to 0.65, indicating that 43% to 65% of the variation in CBARQ scores is explained by $sCBARQ_1$ scores. For subsequent analyses, $sCBARQ_0$ and $sCBARQ_1$ scores were converted into the values predicted by the linear regression formulas in order to make them more comparable to CBARQ scores. Note that homogeneity of variances is an assumption of linear regression and this assumption was not met in our data. However, this violation of the assumptions only has consequences for the test of significance (the p value will be too low). $R^2$, the intercept and the slope will be unbiased (Tate and Wongbundhit 1983).

**Table 6.** Linear regression coefficients for 11 behavioural traits.

| **Factor**[1] | **R square**[2] | **Inter-cept** | **Slope** |
|---|---|---|---|
| SDA | 0.45 | 0.13 | 0.38 |
| ODA | 0.45 | 0.12 | 0.36 |
| AAS[3] | 0.47 | 0.31 | 0.59 |
| EX[3] | 0.47 | 0.68 | 0.51 |
| NSF | 0.58 | 0.21 | 0.55 |
| TRAIN | 0.53 | 1.1 | 0.58 |
| SDF | 0.62 | 0.034 | 0.71 |
| SRB | 0.43 | 0.071 | 0.32 |
| DDA | 0.65 | 0.29 | 0.73 |
| DDF | 0.59 | 0.18 | 0.63 |
| CHASE | 0.60 | 0.38 | 0.65 |

[1] SDA = stranger-directed aggression; ODA=owner-directed aggression; AAS& EX=attachment, attention seeking and excitability; NSF=non-social fear; TRAIN =trainability; SDF=stranger-directed fear; SRB=separation-related behaviours; DDA =dog-directed aggression; DDF=dog-directed fear; CHASE=chasing.
[2] R square=coefficient of determination.
[3] Linear regression was performed separately for AAS and EX because there were two separate $sCBARQ_1$ questions addressing these traits.

The long recall period for $sCBARQ_0$ questions (on average 4.3 years) might result in a decline of the quality of the data (Mathiowetz 2000). We investigated the reliability of $sCBARQ_0$ scores by comparing them with data that were collected in the personal interview with the owner at the time of first participation with Mann-Whitney U or Kruskal-Wallis tests. Dogs that were aggressive towards strangers according to the personal interview had significantly higher $sCBARQ_0$ SDA scores than dogs that were not aggressive towards strangers according to the personal interview ($p<0.0001$; n=115). Similar results were obtained for ODA ($p<0.0001$; n=115) and DDA

($p<0.0001$; $n=117$). We also found a significant difference in $sCBARQ_0$ NSF scores between dogs that were afraid of noises according to the personal interview and those that were not ($p=0.003$; $n=93$). Scores on the $sCBARQ_0$ trainability question also corresponded with the personal interview ($p=0.02$; $n=60$). The better correspondence between personal interview data and aggression scores than between personal interview data and NSF or TRAIN scores was expected because aggressive attacks are very salient for owners and salient events are thought to be less subject to errors of recall decay (Mathiowetz 2000). It was concluded that the five $sCBARQ_0$ scores are reliable in spite of the long recall period.

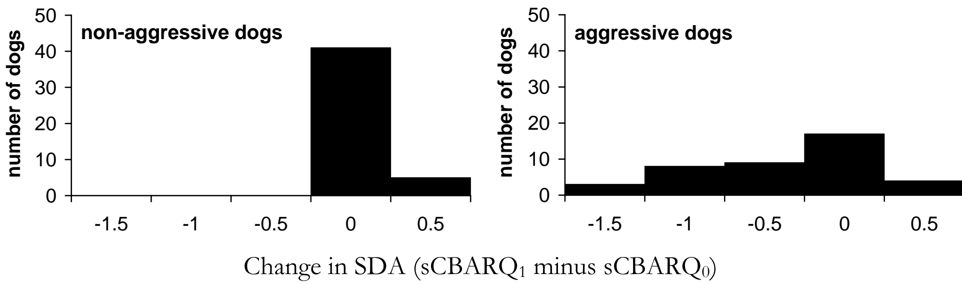
**Behavioural changes in the course of time and their implications for phenotyping**

We analysed changes in aggressive behaviour over time by comparing $sCBARQ_0$ scores to $sCBARQ_1$ scores in the living dogs of group 1. $sCBARQ_0$ scores were subtracted from $sCBARQ_1$ scores; a negative value therefore represents a decrease in aggression in the course of time. We were mainly interested in changes in aggression in dogs that were aggressive in the past. Therefore, we split the group of dogs in two for each analysis: dogs that did not show the particular type of aggression at all in the past (i.e. score = 0; "non-aggressive dogs" in Figure 4) and dogs that showed at least some aggression (score >0; "aggressive dogs" in Figure 4). Twenty-one out of 41 dogs (51%) that showed at least some aggression towards strangers in the past had become less aggressive over time (Figure 4a); 29 out of 39 dogs (74%) that showed some aggression towards their owner had become less aggressive (Figure 4b); 27 out of 49 dogs (55%) that showed some aggression towards other dogs had become less aggressive in the course of time (Figure 4c).

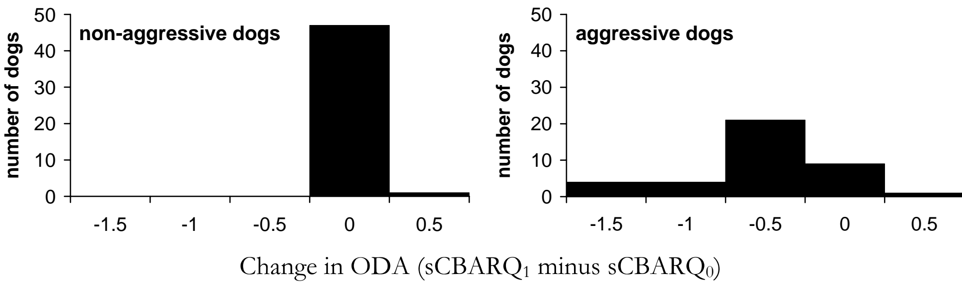
Owners were also asked whether they could explain behavioural changes and 41 owners gave one or several answers to this question. The most frequent answer (28 times) was "I now avoid situations in which problem behaviour is likely to occur.", followed by "I feel I had more control over the dog in the recent past." (13 times). The increased age of the dog was also mentioned several times as an explanation. Owners of deceased dogs of group 1 were also asked whether the behaviour of their dog changed after the first participation. Six owners reported a behavioural change; in five of these dogs the aggressive behaviour had increased and they were euthanatized as a result.

The question is now how to use these findings in our molecular genetic studies. One option is to give more weight to dogs that kept the same level of aggression over time. However, a decrease in the tendency to behave

**a. SDA**




**b. ODA**




**c. DDA**

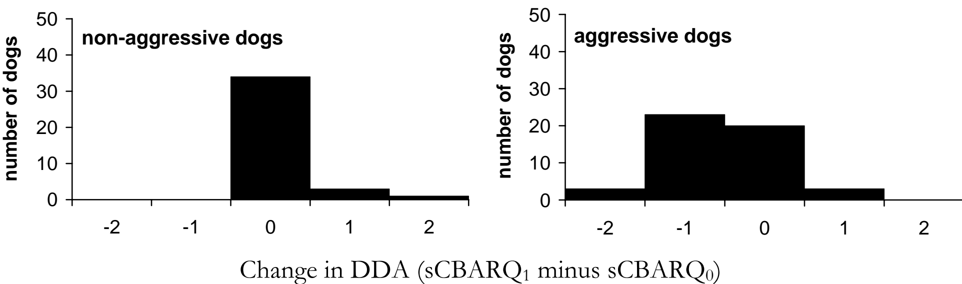



**Figure 4.** Changes in aggressive behaviour over time. a. stranger-directed aggression (SDA) b. owner-directed aggression (ODA) c. dog-directed aggression (DDA). Scores on $sCBARQ_0$ questions were subtracted from $sCBARQ_1$ questions, i.e. negative values represent a decrease in aggressive behaviour. For each type of aggression, the group of dogs was split in two. The left histogram contains the results for dogs that had the lowest possible score on the type of aggression depicted. For example, the left histogram of Figure a represents dogs with a $sCBARQ_0$ stranger-directed aggression score of 0. The right histogram represents dogs that showed at least some aggression, e.g. a $sCBARQ_0$ SDA score of higher than 0.

aggressively does not necessarily mean that genetics plays a lesser role in the aetiology of the aggression of the dogs concerned. The changes might be caused by environmental factors (which is supported by the explanations given

by the owners) and the absence of change may reflect the absence of such environmental forces.

## Shortened CBARQ scores as behavioural phenotypes

Decreases in aggressive behaviour over time are a valid explanation for the low CBARQ aggression scores that we mentioned earlier in this section. We would expect to obtain higher CBARQ aggression scores if we had measured them at the time of first participation. By using $sCBARQ_0$ questions for group 1 dogs and $sCBARQ_1$ questions for group 2 dogs, we can obtain an estimation of CBARQ aggression scores at the first participation. Descriptive statistics of these shortened CBARQ aggression scores are listed in Table 7. As expected, mean shortened SDA, ODA, and DDA scores at first participation were higher than the corresponding mean CBARQ scores (presented in Table 5). Dogs that were recruited as probands scored significantly higher than dogs that were recruited as relatives on all three types of aggression ($p<0.0001$ for SDA, ODA, and DDA; tested with Mann-Whitney U tests).

**Table 7.** Descriptive statistics of shortened CBARQ aggression scores of 228 Golden Retrievers at the time of first participation (st dev=standard deviation).

| | Complete group | | | | | Probands only | | |
|---|---|---|---|---|---|---|---|---|
| **Shortened CBARQ score [1]** | **Number of dogs** | **Minimum** | **Maximum** | **Mean** | **St dev** | **Number of dogs** | **Mean** | **St dev** |
| **SDA** | 228 | 0.13 | 1.7 | 0.46 | 0.46 | 110 | 0.74 | 0.51 |
| **ODA** | 228 | 0.12 | 1.6 | 0.51 | 0.54 | 110 | 0.87 | 0.57 |
| **DDA** | 228 | 0.29 | 3.2 | 1.1 | 0.92 | 110 | 1.5 | 1.0 |

[1] SDA=stranger-directed aggression; ODA=owner-directed aggression; DDA=dog-directed aggression.

## Factor analysis of the aggression test

The factor analysis of CBARQ items resulted in the three aggression types SDA, ODA, and DDA (Table 3). In section 2.1, we analysed the behaviour of 83 Golden Retrievers in an aggression test. Behavioural scores were then

obtained by summing frequencies of aggressive behavioural elements that were displayed in the subtests. We now investigated whether the subtests of the aggression test can be grouped into classes corresponding to CBARQ

**Table 8.** Result of factor analysis on aggression scores in subtest 6-21 of the aggression test for 83 dogs.

| Subtest | Factor | | | Commu-nality |
|---|---|---|---|---|
| | **DDA[1] (23.2%)** | **SDA[1] (20.7%)** | **PA[1] (20.3%)** | |
| **6 (squeezing groins)** | 0.79 | | | 0.64 |
| **7 (pull feeding bowl by test person)** | | | 0.81 | 0.66 |
| **8 (pull feeding bowl by owner)** | | | 0.88 | 0.79 |
| **9 (umbrella)** | | 0.64 | | 0.42 |
| **10 (strange woman)** | | 0.81 | | 0.72 |
| **11 (clapping)** | | 0.67 | | 0.52 |
| **12 (hitting)** | | 0.59 | *0.54* | 0.64 |
| **13 (cornering)** | | *0.39* | 0.64 | 0.56 |
| **14 (threatening the owner)** | | *0.38* | 0.81 | 0.85 |
| **15 (cornering with dogs)** | 0.79 | | | 0.64 |
| **16 (dominant dog)** | 0.78 | | *0.39* | 0.76 |
| **17 (owner pets other dog)** | 0.71 | | | 0.57 |
| **18 (feeding in presence of other dog)** | 0.74 | | *0.41* | 0.72 |
| **19 (feeding bowl given to other dog)** | 0.75 | | | 0.57 |
| **20 (doll)** | | 0.83 | | 0.69 |
| **21 (dog mask)** | *0.39* | 0.62 | | 0.54 |
| **Cronbach's α** | 0.77 | 0.80 | 0.79 | |

[1] Numbers in brackets represent the percentage of variance explained by the factors. Loadings between –0.3 and 0.3 are not shown and cross-loadings are printed in italics. DDA=dog-directed aggression; SDA=stranger-directed aggression; PA=possessive aggression.

aggression types by performing factor analysis on aggression scores per subtest. Three factors were extracted, explaining 64.2% of the variance in subtest scores (Table 8). The first factor contained subtest 15-19, which involved confrontations of the Golden Retriever with other dogs. The factor was labelled "dog-directed aggression". Subtest 6 (squeezing groins) also loaded on this factor. Subtests 9-12, 20, and 21 loaded high on the second factor. These subtests involve confrontations with strangers and the factor was

thus labelled "stranger-directed aggression". The third factor contained items 7 (pull feeding bowl by test person), 8 (pull feeding bowl by owner), 13 (cornering), and 14 (threatening the owner). Item 18 (feeding in presence of other dog) correlated with the third factor and item 12 (hitting) loaded approximately equally on factor two and three. This third factor was labelled "possessive aggression". Cronbach's alpha values for the factors were 0.77 (0.79 when subtest 6 was excluded), 0.80, and 0.79 respectively.

The first two factors that we extracted closely resemble the two CBARQ factors dog-directed aggression and stranger-directed aggression in spite of the fact that we did not select subtests for their comparability to CBARQ items. This provides additional support for the reliability of these CBARQ factors. Subtest 6 (squeezing groins) unexpectedly loaded on the dog-directed aggression factor. We expected this subtest to represent owner-directed aggression, so correlations with the third factor would seem more logical. This subtest might be comparable to "pain sensitivity" CBARQ items, which failed to behave consistently in our factor analysis of the CBARQ items.

The third type of aggression in CBARQ, owner-directed aggression, was not found in the aggression test dataset. We did find a factor containing food-related subtests and subtest 13 (cornering) and 14 (threatening the owner). We interpreted aggressive behaviour of the dogs in the latter two subtests as protection of the owner. In functional classifications of canine aggression, possessive (or food-related) and protective (or territorial) aggression are distinguished (Borchelt and Voith 1996; Landsberg *et al.* 2004; Reisner 2003). Borchelt (1983) mentioned correlations between these aggression classes in clinical datasets. The present results confirm the presence of this correlation empirically.

The factor analysis of CBARQ items resulted in the three fear types SDF, NSF, and DDF (Table 3). A factor analysis was performed on fear scores per subtest in order to find out whether subtests of the aggression test can be grouped into classes corresponding to CBARQ fear types. Two factors explaining 70% of the variance in subtest scores were extracted (Table 9). The first factor contained subtest 6, 9-17, and 19-21, while the second factor contained subtest 7 and 8 (food bowl), 17 (owner pets other dog), and 18 (feeding in presence of other dog). Subtests loading high on the first factor seem to involve threatening of the dog and the factor was labelled "threatening subtests". The second factor contains more neutral stimuli and was thus labelled "non-threatening subtests". Cronbach's alpha values for the factors were 0.96 and 0.67 (including subtest 17) respectively.

The finding of three separate aggression test factors suggests that some dogs showed more aggression in specific groups of subtests than in others. A

similar specialisation was not observed when fear scores per subtest were analysed. Here, we found a subdivision in threatening and non-threatening subtests. The dogs apparently did not make a consistent distinction between different types of stimuli (i.e. dogs or humans); dogs were either fearful or not fearful in all of these subtests. We expect this to be an artefact of the test: fearful behaviour in the test might be a reflection of how impressed the dogs are by the test situation.

**Table 9.** Result of factor analysis on fear scores in subtest 6-21 of the aggression test for 83 dogs.

| | **Factor** | | |
|---|---|---|---|
| **Subtest** | **THREAT[1] (54.9%)** | **NO THREAT[1] (15.0%)** | **Communality** |
| **6 (squeezing groins)** | 0.83 | | 0.69 |
| **7 (pull feeding bowl by test person)** | | 0.82 | 0.69 |
| **8 (pull feeding bowl by owner)** | | 0.73 | 0.60 |
| **9 (umbrella)** | 0.90 | | 0.84 |
| **10 (strange woman)** | 0.79 | *0.37* | 0.76 |
| **11 (clapping)** | 0.88 | | 0.77 |
| **12 (hitting)** | 0.79 | | 0.63 |
| **13 (cornering)** | 0.85 | | 0.74 |
| **14 (threatening the owner)** | 0.84 | | 0.79 |
| **15 (cornering with dogs)** | 0.87 | *0.31* | 0.86 |
| **16 (dominant dog)** | 0.78 | | 0.69 |
| **17 (owner pets other dog)** | 0.57 | *0.54* | 0.61 |
| **18 (feeding in presence of other dog)** | | 0.64 | 0.42 |
| **19 (feeding bowl given to other dog)** | 0.79 | | 0.63 |
| **20 (doll)** | 0.87 | | 0.78 |
| **21 (dog mask)** | 0.83 | | 0.69 |
| **Cronbach's α** | 0.96 | 0.67 | |

[1] Numbers in brackets represent the percentage of variance explained by the factors. Loadings between –0.3 and 0.3 are not shown and cross-loadings are printed in italics. THREAT= threatening subtests; NO THREAT=non-threatening subtests.

## Comparison of aggression test scores with $sCBARQ_0$ scores

Three aggression test scores were calculated by summing aggression scores during subtests of each factor (e.g. “dog-directed aggression test score” = sum of aggression scores in subtest 15-19). We calculated Spearman’s rank correlation coefficients for every possible combination of the three aggression test scores and the three $sCBARQ_0$ questions about aggression. We expected to find positive correlations between the stranger-directed aggression test score and the $sCBARQ_0$ question about SDA and between the dog-directed aggression test score and the $sCBARQ_0$ question about DDA. We might also expect a positive correlation between the possessive aggression test score and the $sCBARQ_0$ question about ODA, because some of the subtests of the possessive aggression factor resembled CBARQ items about ODA. All these correlations were found indeed (diagonal in Table 10). Lower but still significant positive correlations were found between aggression test scores and the non-corresponding $sCBARQ_0$ scores (off-diagonal values in Table 10). In other words, there was significant convergent validity (evidence of similarity between measures of theoretically related constructs), but poor discriminant validity (absence of correlation between measures of unrelated constructs) between aggression test scores and $sCBARQ_0$ scores.

We also found a significant agreement between owner-acknowledged information and aggression test scores in our previous study, in spite of the fact that there were many owner-acknowledged aggressive dogs that showed

**Table 10.** Spearman’s rank correlation coefficients of three aggression test scores (in rows) and three $sCBARQ_0$ aggression scores (in columns) from 70 dogs.

| **Aggression test score**[2] | **$sCBARQ_0$ SDA score**[1] | **$sCBARQ_0$ ODA score**[1] | **$sCBARQ_0$ DDA score**[1] |
|---|---|---|---|
| **SDA** | 0.43* | 0.26# | 0.25# |
| **PA** | 0.42* | 0.36* | 0.25# |
| **DDA** | 0.34* | 0.30# | 0.45* |

[1] SDA=stranger-directed aggression; ODA=owner-directed aggression; DDA=dog-directed aggression.

[2] SDA=stranger-directed aggression in the aggression test; PA=possessive aggression in the aggression test; DDA=dog-directed aggression in the aggression test.

* Correlation is significant at the 0.01 level (2-tailed).

# Correlation is significant at the 0.05 level (2-tailed).

little aggression in the test (section 2.1). In that study, behavioural scores were obtained by summing frequencies of aggressive behavioural elements that were displayed in the subtests and the owner-acknowledged information was derived from the personal interviews. Svartberg (2005) found no significant correlation between aggression in the Swedish Dog Mentality Assessment and the stranger-directed aggression CBARQ score in his dogs. It is hard to comment on the disagreement between our findings since the two studies cannot be compared because we used different behavioural tests, different questionnaires, and different subjects.

Several explanations can be given for the poor discriminant validity. First and most importantly, as we already showed in the paragraph about variation in CBARQ scores, there frequently was a co-occurrence of different types of aggression within single dogs. This will result in indirect correlations between seemingly unrelated shortened CBARQ questions and aggression test scores. Second, the aggressive behaviour observed in the aggression test might be a reflection of aggressiveness in unfamiliar situations, regardless of the exact type of stimulus involved. This explanation is line with the suggestion of Svartberg (2005) that aggression in the Swedish Dog Mentality Assessment reflects aggression towards novel stimuli. However, if aggression in our test would merely reflect aggression towards novel stimuli, we would not expect to find three different groups of subtests in the factor analysis. We would rather expect to find only one major aggression factor, similar to the one major fear factor. This hypothesis can therefore at most partially explain the poor discriminant validity.

The results presented above highlight two disadvantages of using aggression tests as a method for phenotyping. First, it seems that we were not able to elicit an important class of aggression in the test: owner-directed aggression. At first glance, the possessive aggression factor seems to include ODA, but Table 10 shows that possessive aggression test scores in fact correlated better with SDA in the CBARQ than with ODA. On logical grounds, it is to be expected that territorial aspects of SDA cannot be elicited in an artificial situation either. Second, fearful behaviour in the aggression test was not object-specific. This suggests that fearful behaviour in the test is a reflection of fear in unfamiliar situations and this may apply partially to aggression in the test as well. This may also explain our earlier observation that many Golden Retrievers that were aggressive according to their owner showed little or no aggression in the test (section 2.1). We can conclude that, in spite of the positive correlations between aggression test scores and shortened CBARQ scores, the behaviour of the dogs in the aggression test is likely to be not completely representative of their behaviour in everyday life.

## Conclusion

In summary, factor analysis of CBARQ items revealed a nearly identical factor structure as found in previous studies. The internal consistency reliability of the factors was high. In addition, two out of three CBARQ aggression factors could also be found in the aggression test data that we collected earlier. CBARQ aggression scores displayed substantial variation within the Golden Retriever families, which is a prerequisite for genetic studies. Thus, the behavioural scores derived from the CBARQ provide a promising tool for future research. We consider the CBARQ a more useful instrument for phenotyping than the aggression test because the CBARQ encompasses a higher number of everyday life situations and because behaviour in the aggression test may not be representative of the behaviour of the dog in everyday life. Genetic parameters of various CBARQ based behavioural scores will now be investigated in quantitative genetic studies. These studies will give more insight into the usefulness of the scores for molecular genetic studies.

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**Appendix I.** The canine behavioural assessment and research questionnaire

| Category | | CBARQ item | |
|---|---|---|---|
| TRAINING AND OBEDIENCE | Dog | 1 | returns immediately when called when off leash. |
| | | 2 | obeys the "sit" command immediately. |
| | | 3 | obeys the "stay" command immediately. |
| | | 4 | seems to attend / listen closely to everything you say or do. |
| | | 5‡ | is slow to respond to correction or punishment ("thick-skinned"). |
| | | 6‡ | is slow to learn new tricks or tasks. |
| | | 7‡ | is easily distracted by interesting sights, sounds, or smells. |
| | | 8 | will retrieve (or attempt to retrieve) sticks, balls, or objects. |
| AGGRESSION | Dog acts aggressively | 9 | when verbally corrected or punished (scolded, shouted at, etc) by you or a household member. |
| | | 10# | when approached directly by an unfamiliar adult while being walked / exercised on a leash. |
| | | 11 | when approached directly by an unfamiliar child while being walked / exercised on a leash. |
| | | 12 | toward unfamiliar persons approaching the dog while s/he is in your car (at a parking lot for example). |
| | | 13 | when toys, bones or other objects are taken away by household memb. |
| | | 14 | when bathed or groomed by a household member. |
| | | 15 | when an unfamiliar person approaches you or another member of your family at home. |
| | | 16 | when unfamiliar persons approach you or another member of your family away from your home. |
| | | 17 | when approached directly by a household member while s/he is eating. |
| | | 18 | when mailmen or other delivery workers approach your home. |
| | | 19 | when his/her food is taken away by a household member. |
| | | 20 | when strangers walk past your home while your dog is in the yard. |
| | | 21* | when an unfamiliar person tries to touch or pet the dog. |
| | | 22 | when joggers or cyclists pass your home while your dog is in the yard. |
| | | 23 | when approached directly by an unfamiliar male dog while being walked or exercised on a leash. |
| | | 24 | when approached directly by an unfamiliar female dog while being walked or exercised on a leash. |
| | | 25 | when stared at directly by a member of the household. |
| | | 26 | toward unfamiliar dogs visiting your home. |
| | | 27 | toward cats or other animals entering your yard. |
| | | 28 | toward unfamiliar persons visiting your home. |
| | | 29* | when barked, growled or lunged at by another (unfamiliar) dog. |
| | | 30 | when stepped over by a member of the household. |
| | | 31 | when you or a household member retrieves food or objects stolen by the dog. |
| | | 32* | towards another (familiar) dog in your household. |
| | | 33* | when approached at a favourite resting / sleeping place by another (familiar) household dog. |
| | | 34* | when approached while eating by another (familiar) household dog. |
| | | 35* | when approached while playing with / chewing a favourite toy, bone, object, etc., by another (familiar) household dog. |

## Appendix I, continued

| Category | Stem | No. | Item |
|---|---|---|---|
| FEAR AND ANXIETY | Dog acts anxious or fearful | 36# | when appr. directly by an unfamiliar adult while away from your home. |
| | | 37 | when appr. directly by an unfamiliar child while away from your home. |
| | | 38 | in response to sudden or loud noises (e.g. vacuum cleaner, car backfire, road drills, objects being dropped, etc.). |
| | | 39 | when unfamiliar persons visit your home. |
| | | 40* | when an unfamiliar person tries to touch or pet the dog. |
| | | 41 | in heavy traffic. |
| | | 42 | in response to strange or unfamiliar objects on or near the sidewalk (e.g. plastic trash bags, leaves, litter, flags flapping, etc.). |
| | | 43 | when examined or treated by a veterinarian. |
| | | 44 | during thunderstorms. |
| | | 45 | when appr. directly by an unfamiliar dog of the same or larger size. |
| | | 46 | when approached directly by an unfamiliar dog of a smaller size. |
| | | 47 | when first exposed to unfamiliar situations (e.g. first car trip, first time in elevator, first visit to veterinarian, etc.). |
| | | 48 | in response to wind or wind-blown objects. |
| | | 49 | when having claws clipped by a household member. |
| | | 50 | when groomed or bathed by a household member. |
| | | 51* | when having his/her feet towelled by a member of the household. |
| | | 52* | when unfamiliar dogs visit your home. |
| | | 53* | when barked, growled or lunged at by another unfamiliar dog. |
| SEPARATION-RELATED BEHAVIOUR | When left or about to be left on its own, the dog displays | 54 | shaking, shivering or trembling. |
| | | 55 | excessive salivation. |
| | | 56 | restlessness / agitation / pacing. |
| | | 57 | whining. |
| | | 58 | barking. |
| | | 59 | howling. |
| | | 60 | chewing / scratching at doors, floor, windows, curtains, etc. |
| | | 61 | loss of appetite. |
| EXCITABILITY | Dog overreacts or is excitable | 62 | when you or household members come home after a brief absence. |
| | | 63 | when playing with you or other members of your household. |
| | | 64 | when doorbell rings. |
| | | 65 | just before being taken for a walk. |
| | | 66 | just before being taken on a car trip. |
| | | 67 | when visitors arrive at your home. |
| ATTACHMENT AND ATTENTION SEEKING | Dog | 68 | displays a strong attachment for one particular household member. |
| | | 69 | tends to follow you or household members about the house. |
| | | 70 | tends to sit close to, or in contact with, you when you are sitting. |
| | | 71 | tends to nudge, nuzzle, or paw for attention when you are sitting. |
| | | 72 | becomes agitated (whines, jumps up, tries to intervene) when you (or others) show affection for another person. |
| | | 73 | becomes agitated (whines, jumps up, tries to intervene) when you (or others) show affection for another dog or animal. |
| MISCEL-LA-NEOUS | Dog chases | 74 | cats (given the chance). |
| | | 75 | birds (given the chance). |
| | | 76 | other animals (e.g. rabbits) (given the chance). |

# Two items in the PennBARQ were fused to create this item. The PennBARQ is an earlier version of the CBARQ (Hsu and Serpell 2003).
* Item was not present in the PennBARQ (Hsu and Serpell 2003).
‡ Scores on this item were reversed, i.e. never = 4, seldom = 3, etc.

## **Appendix II.** Shortened CBARQ questions

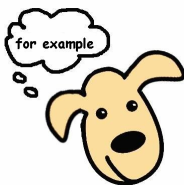
We designed a shortened version of the Canine behavioural assessment and research questionnaire (sCBARQ), in which only one question addressed each of the Hsu and Serpell factors (2003). These sCBARQ questions were developed with two aims. First, we wanted to use a relatively short questionnaire to obtain behavioural information about deceased dogs because we considered it inappropriate to send a long questionnaire to owners of deceased dogs. Second, we wanted to use the shortened CBARQ questions to study behavioural changes of the dogs in the course of time.

Two types of shortened CBARQ questions were made: questions that addressed the behaviour of the dog in the months prior to its first participation in the project (sCBARQ$_0$ questions) and questions that addressed the behaviour of the dog in the recent past (sCBARQ$_1$ questions). The questions listed here are of the sCBARQ$_0$ type. The sCBARQ$_0$ questions were introduced with two instrumental questions in order to help the owners to search their memory (e.g. "In what parts of our project did you participate?").

---

The following questions address to your dog's behaviour at the time you participated in our project for the first time. Please think back the months *preceding your visit to our clinic*. In most questions there are examples next to the "thinking dog" to help you fill out the questionnaire. It is likely that you will recognize your dog better in some examples than in others. We ask you to choose the answer that *on average* describes your dog's behaviour the best. We ask you *not* to base your answers on one single example.

**1.** Most dogs are strongly attached to their owner. Some demand a great deal of attention.

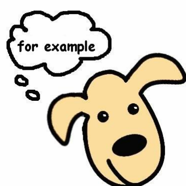



For instance:
- Does your dog display strong attachment for one particular household member?
- Does the dog tend to follow household members about the house?
- Does the dog seek attention when you or a family member sit somewhere (sit close to you; nudge, nuzzle or paw you)?
- Does your dog become agitated when you show affection for another person or animal (whining, jumping up, trying to intervene)?

It is possible that your dog displays strong attachment for one particular member of the household, while it does not follow people about the house. In this case, please try to choose the answer that *in general* describes your dog the best. By checking the appropriate box, please indicate how attached your dog was at that time and how often it sought attention.

| NEVER | SELDOM | SOMETIMES | USUALLY | ALWAYS |
|---|---|---|---|---|
| ☐ | ☐ | ☐ | ☐ | ☐ |

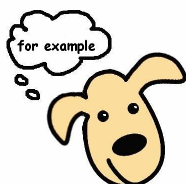
**2.** Some dogs show signs of anxiety or abnormal behaviour when left alone, even for relatively short periods of time. How often did your dog show signs of separation-related behaviour at the time? (Check the box that describes your dog the best.)




A dog with separation-related problems may display the following behaviour when left, or about to be left, on its own:
- trembling, restlessness;
- whining, barking, howling;
- chewing or scratching at doors, windows, etc.;
- loss of appetite, excessive salivation.

| NEVER | SELDOM | SOMETIMES | USUALLY | ALWAYS |
|---|---|---|---|---|
| ☐ | ☐ | ☐ | ☐ | ☐ |

**3.** Some dogs are more obedient and trainable than others. By checking the appropriate box, please indicate how trainable or obedient your dog was at that time.




For instance:
- Did your dog immediately obey commands?
- Was your dog fast to learn new tricks?
- Did your dog retrieve objects?
- Was your dog fast to respond to correction?
- Did your dog attend closely to what you did and was it not easily distracted?

| NEVER | SELDOM | SOMETIMES | USUALLY | ALWAYS |
|---|---|---|---|---|
| ☐ | ☐ | ☐ | ☐ | ☐ |

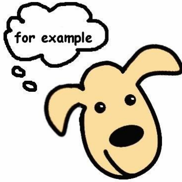
**4.** Did your dog chase birds, cats, or other animals in the months preceding your visit to our clinic (given the chance)?

| NEVER | SELDOM | SOMETIMES | USUALLY | ALWAYS |
|---|---|---|---|---|
| ☐ | ☐ | ☐ | ☐ | ☐ |

The next three questions deal with aggressive behaviour. Some dogs display aggressive behaviour from time to time. Typical signs of moderate aggression include barking, growling and baring teeth. More serious aggression generally includes snapping, lunging, biting, or attempting to bite.

Please *circle* the number that describes your dog the best in the following three questions. With each question you can choose between five answers: 0= no aggression, 4=serious aggression and 1-3 are in between.

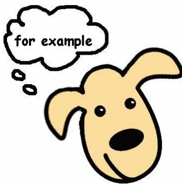
**5.** What was your dog's tendency to display aggressive behaviour toward *unfamiliar persons* at that time?




For instance:
- When approached or petted by a stranger (adult or child).
- When a stranger approached you or a family member.
- When a stranger visited your home.
- When strangers walked by your home (e.g. mailmen)

| No aggression: no visible signs of aggression | Moderate aggression: growling/ barking/ baring teeth<br>**0.......1.......2.......3.......4** | Serious aggression: snapping, biting or attempting to bite |
|---|---|---|

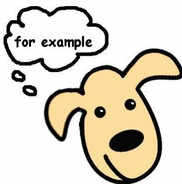
**6.** What was your dog's tendency to display aggressive behaviour toward *familiar persons* at the time?




For instance, consider these situations in which you or a family member …
- verbally corrected your dog.
- took away food or a toy from your dog.
- approached the dog while s/he was eating.
- bathed or groomed the dog.
- stepped over the dog or stared at the dog.

| No aggression: no visible signs of aggression | Moderate aggression: growling/ barking/ baring teeth<br>**0.......1.......2.......3.......4** | Serious aggression: snapping, biting or attempting to bite |
|---|---|---|

**7.** What was your dog's tendency to display aggressive behaviour toward *unfamiliar dogs* at that time?



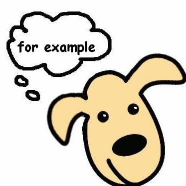

For instance, consider these situations in which an unfamiliar dog
- directly approached your dog while being walked on a leash.
- visited your home.
- growled, barked or lunged at your dog.

| No aggression: no visible signs of aggression | Moderate aggression: growling/ barking/ baring teeth<br>**0.......1.......2.......3.......4** | Serious aggression: snapping, biting or attempting to bite |
|---|---|---|

The next three questions deal with fear and anxiety. Dogs sometimes show signs of anxiety or fear when exposed to particular sounds, objects, or persons. Typical signs of moderate fear include: avoiding eye contact, avoiding the feared object, crouching or cringing with tail lowered or tucked between the legs, whining, whimpering, freezing, shaking, and trembling. Extreme fear is characterized by exaggerated cowering and/or vigorous attempts to escape, retreat or hide from the feared object, person or situation.

Would you please circle the number that describes your dog best in the following three questions? (0= no fear; 4 = extreme fear; 1-3 = in between)

**8.** What was your dog's tendency to display fear of *unfamiliar persons* at that time?

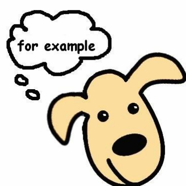



For instance:
- When a stranger (adult or child) approached your dog or tried to pet it.
- When a stranger visited your home.

Mild - moderate fear

No fear: no visible signs of fear **0.......1.......2.......3.......4** Extreme fear: cowering, retreating, hiding, etc.

**9.** What was your dog's tendency to display fear of *unfamiliar dogs* at the time?

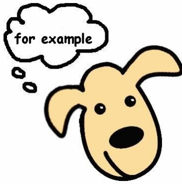



For instance, consider these situations when an unfamiliar dog
- approached your dog directly.
- visited your home.
- growled, barked or lunged at your dog.

Mild - moderate fear

No fear: no visible signs of fear **0.......1.......2.......3.......4** Extreme fear: cowering, retreating, hiding, etc.

**10.** What was your dog's tendency to display fear of *unfamiliar objects and sounds* at that time?



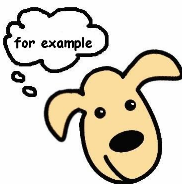

For instance:
- sudden or loud noises such as objects being dropped, the vacuum cleaner and road drills.
- heavy traffic or thunderstorms.
- outside: plastic trash bags, leaves, litter, wind-blown objects, flags flapping.
- when first exposed to an unfamiliar situation (elevator, car, veterinarian).

Attention! Please do not rely on just one of these examples. Just like with the previous questions, we ask you to choose the number that describes your dog best *in general*.

| No fear: no visible signs of fear | Mild - moderate fear<br>**0.......1.......2.......3.......4** | Extreme fear: cowering, retreating, hiding, etc. |
|---|---|---|

**11.** Some dogs show little reaction to sudden or potentially exciting events. Others become highly excited at the slightest novelty. Signs of moderate excitability include increased alertness, movement towards the source of novelty, and brief episodes of barking. Extreme excitability is characterized by a general tendency to over-react. The dog barks or yelps hysterically at the slightest disturbance, rushes towards and around any source of excitement, and is difficult to calm down.

Using the scale beneath the "thinking dog", please indicate your own dog's tendency to become excitable at the time (0=calm; 4=extremely excitable; 1-3=in between).




For instance:
- When you or a family member came home.
- When you or a family member played with the dog.
- When the doorbell rang.
- Just before being taken on a walk or on a car trip.
- When visitors arrived at your home.

| Calm: little or no special reaction | Mild – moderate excitability<br>**0…......1…….2…….3…….4** | Extremely excitable: over-reacts, hard to calm down |
|---|---|---|

# 2.3
# Genetic variation in aggression related traits in Golden Retrievers

Anna-Elisa Liinamo, Linda van den Berg, Peter A.J. Leegwater, Matthijs B.H. Schilder, Johan A.M. van Arendonk, and Bernard A. van Oost

This section has been submitted for publication.

## Abstract

In this study, heritabilities of several measures of aggression were estimated in a group of 325 Golden Retrievers, using the restricted maximum likelihood method. The studied measures were obtained either through owner opinions or by using the canine behavioural assessment and research questionnaire (CBARQ). The aim of the study was to determine which of the aggression measures showed sufficient genetic variation to be useful as phenotypes for future molecular genetic studies on aggression in this population. The most reliable heritability estimates seemed to be those for simple dog owner impressions of human- and dog-directed aggression, with heritability estimates of 0.77 (s.e. 0.09) and 0.81 (s.e. 0.09), respectively. In addition, several CBARQ derived measures related to human-directed aggression showed clear genetic differences between the dogs. The relatively low correlation between the estimated breeding values for owner impressions on human- and dog-directed aggression suggests that these two traits have a partially different genetic background. They will therefore have to be treated as separate traits in further genetic studies.

## Introduction

According to the breed standard, Golden Retriever dogs should have an ideal character to make good family pets. For example, the Dutch Golden Retriever Club breed standard states that Golden Retrievers are "good-natured, friendly, and confident" (http://www.goldenretrieverclub.nl/ ; link accessed August 2005). However, there are also reports of very aggressive Golden Retrievers (Edwards 1991; Galac and Knol 1997; Heath 1991; Knol and Schilder 1999). A genetic background has been suggested for this aggression because the behaviour seems to occur more often in certain Golden Retriever family groups (Knol *et al.* 1997). Consequently, a study of the genetics of aggressive behaviour in Golden Retrievers was started at Utrecht University in 1997. The aim of this project is to identify mutations or polymorphisms in genes underlying the variation in aggressive behaviour in Golden Retrievers.

The key to success in any genetic study is the availability of a reliable and valid method for phenotyping. Obtaining phenotypes is especially challenging for behavioural traits, because of their complexity with many contributing factors (e.g. Mackenzie *et al.* 1986). Two main methods exist for phenotyping canine aggression: behavioural tests and questionnaires. In behavioural tests dogs are evaluated for their responses towards various stimuli under controlled conditions by trained evaluators. When using questionnaires, dog owners are asked opinions on the behaviour of their dog in various everyday situations. Examples of the first method include the aggression test developed by Netto and Planta (1997), and of the latter method the canine behavioural assessment and research questionnaire (CBARQ) developed by Hsu and Serpell (2003). Data obtained with either method have thus far not been studied genetically, even though the aggression test was specifically developed for excluding aggressive individuals from breeding, and the CBARQ has been suggested to be a useful research tool for exploring the causes of behavioural problems in dogs.

In theory, behavioural tests are more objective than owner-derived information. We studied the usefulness of a shortened version of the aggression test of Netto and Planta (1997) as a means of phenotyping aggression in Golden Retrievers in section 2.1 and 2.2 of this thesis. These studies concluded that certain types of aggression cannot be elicited in the test and that the behaviour in a test may not be representative of the behaviour of the dog in everyday life. It is thus questionable if the aggression test could be used to reliably classify dogs into aggression classes for genetic analyses in this population. In contrast, the behavioural scores derived from the CBARQ seem to provide a promising tool for genetic studies of aggression in the Golden

Retriever population (section 2.2). However, before progressing to more complex and costly molecular genetic analyses it is important to determine the degree of heritability, i.e. the strength of the relationship between the genetic and phenotypic values, for the CBARQ scores in this population.

The aim of this paper was to estimate heritabilities of aggression related measures obtained via the CBARQ on a population of Golden Retrievers in The Netherlands. More precisely, the goal was to study which measures of aggression that are available from the CBARQ show sufficient genetic variation to be interesting candidates for further molecular genetic studies on canine aggression in this population. We also calculated the Pearson product moment correlation coefficient between the estimated breeding values for the most reliable human- and dog-directed aggression measures to provide an impression of the extent to which these two traits are genetically related to each other.

## Materials and methods

### Subjects

The data analysed in this study were collected between 1997 and 2005 with the main target being family-based molecular genetic studies on aggression in the Dutch Golden Retriever population. It concerns 325 Golden Retrievers of which we obtained varying types of behavioural information. The dogs were recruited to the project either because of their aggressive behaviour (probands, n=159) or because they were related to an aggressive dog (siblings and parents, n=166). Of the 325 dogs that had phenotypes available, 241 had a known pedigree containing at least three generations, through which 865 additional ancestral dogs without own phenotypes could be traced.

The phenotypic data included offspring of 108 known sires, with on average 2.3 offspring per sire (Figure 1). The largest sire progeny group consisted of 14 offspring (including both full- and half-sibs), but the majority of sires only had one phenotyped offspring. These sires had been mated with 125 known dams, which had on average 1.9 offspring in the data (Figure 2). Like the sires, most dams had one phenotyped offspring. The largest dam progeny group consisted of 10 offspring including both full-and half-sibs. Most of the other dam progeny groups consisted of only full-sibs. For 84 phenotyped dogs pedigree data was not available. The majority of the other dogs were connected with other families in the data through their pedigrees.

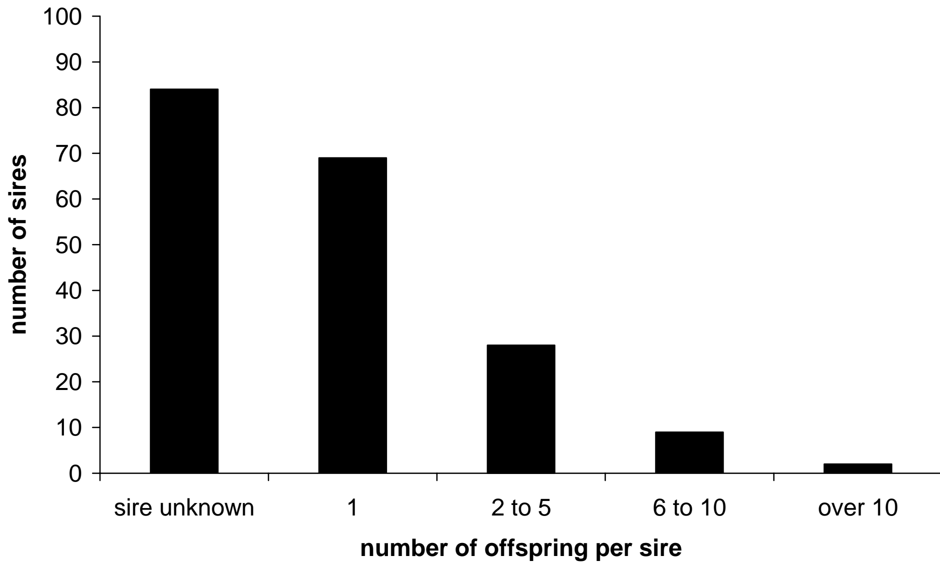



**Figure 1.** Distribution of phenotyped offspring over sires.

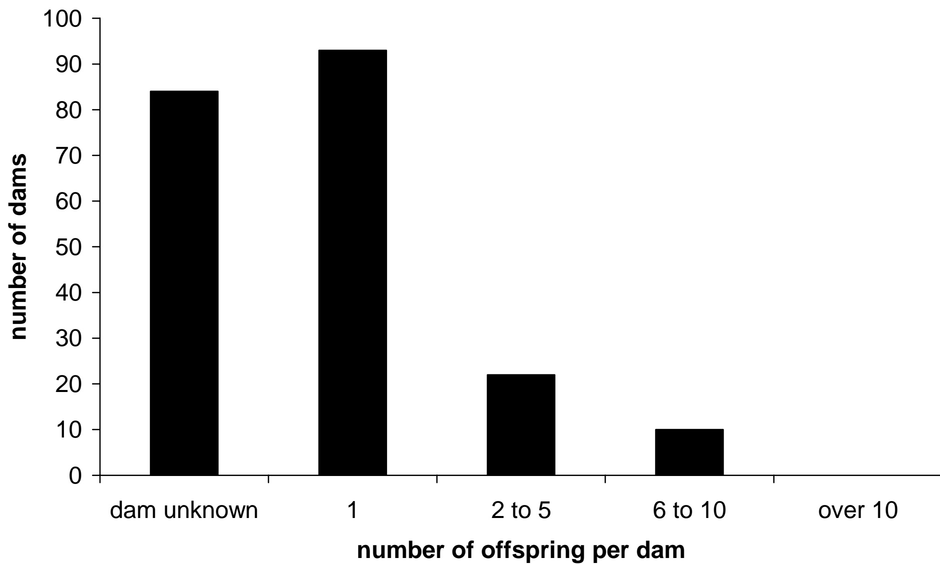



**Figure 2.** Distribution of phenotyped offspring over dams.

## Behavioural measures

Four types of behavioural measures were analysed in this study. The first type was the owner impression. When a dog was recruited to the study, its owner was asked for his or her opinion on the aggressiveness of the dog (this is part

of the "personal interview" that is mentioned in section 2.1 and 2.2). Owners were asked if their dog was aggressive towards humans and if it was aggressive towards other dogs. The status of the dog was coded in three classes: non-aggressive (score 1), threatens (score 2), or bites (score 3). Almost all phenotyped dogs had the owner impression available on both human- and dog-directed aggression (Table 1).

Items of the CBARQ were the second type of behavioural measure that we analysed. Most of the dog owners were asked to fill out the CBARQ. In contrast to the owner impressions, many owners completed the CBARQ several years after their first participation in the study (section 2.2). The questionnaire included 27 items on the aggressiveness of the dog in various everyday situations (for more info on the CBARQ, see Hsu and Serpell, 2003). CBARQ items were scored with 5-point qualitative rating scales (i.e. 0 = no signs of aggression; 1 to 3 = moderate aggression, i.e. growling and baring teeth; and 4 = serious aggression, i.e. snapping and biting). The majority of the dogs had CBARQ data available on all aggression related items. The exception was aggression related to the behaviour of the dog towards other dogs in the same family, because only owners with multi-dog households could answer these items (Table 1).

So-called "shortened CBARQ scores" were the third type of behavioural measure that we analysed. Hsu and Serpell (2003) demonstrated by means of factor analysis that CBARQ aggression items can be grouped into stranger-directed aggression, owner-directed aggression, and strange dog-directed aggression. Consequently, the owners were asked to give answers to "shortened CBARQ questions", where each question addressed one Hsu and Serpell factor directly (for more information on the shortened CBARQ questions and the reason why they were designed, see section 2.2). The shortened CBARQ questions that we used here addressed the behaviour of the dog in the months prior to its first participation in the project. The shortened questions were therefore similar to the owner impressions, except that they separated human-directed aggression in aggression towards strangers and owners and that they were evaluated on a 5-point scale similar to the original CBARQ items. Shortened CBARQ questions were further converted into "shortened CBARQ scores" predicted by linear regression formulas that were described in section 2.2. These formulas were obtained by means of linear regression of CBARQ scores on shortened CBARQ questions. (CBARQ scores were calculated for the three aggression-related factors as the mean of the CBARQ items that were grouped in a factor by Hsu and Serpell; see section 2.2) This was done to make the shortened CBARQ scores easier to compare with the original CBARQ scores. Answers to these questions were

**Table 1.** Abbreviations, descriptive statistics, and heritability estimates of 36 studied aggression measures. (N=number of observations; SD= standard deviation; CV%=coefficient of variation; Min=minimum value; Max=maximum value; $h^2$=heritability; se=standard error of $h^2$ estimate).

| | Abbre viation | Descriptives | | | | | | Heritability estimate | |
|---|---|---|---|---|---|---|---|---|---|
| **Aggression measure** | | **N** | **Mean⁺** | **SD** | **CV%** | **Min⁺** | **Max⁺** | **$h^2$** | **se** |
| *Owner impressions:* | | | | | | | | | |
| Human-directed aggression | haggr | 316 | 1.85 | 0.93 | 50 | 1 | 3 | 0.77 | 0.09 |
| Dog-directed aggression | daggr | 312 | 1.55 | 0.82 | 53 | 1 | 3 | 0.81 | 0.09 |
| *Original CBARQ items:* | | | | | | | | | |
| Verbal correction by owner | cb9 | 217 | 1.25 | 0.68 | 54 | 1 | 5 | 0.11 | 0.05 |
| Strange adult approaching leashed dog | cb10 | 217 | 1.24 | 0.61 | 49 | 1 | 4 | 0.85 | na* |
| Strange child approaching leashed dog | cb11 | 216 | 1.29 | 0.74 | 57 | 1 | 5 | 1.00 | na* |
| Stranger approaching dog in car | cb12 | 206 | 1.38 | 0.92 | 67 | 1 | 5 | 0.07 | 0.09 |
| Family member removing toy | cb13 | 217 | 1.48 | 1.02 | 69 | 1 | 5 | 0.32 | 0.17 |
| Family member grooming dog | cb14 | 215 | 1.22 | 0.64 | 52 | 1 | 5 | 0.83 | na* |
| Stranger approaching owner at home | cb15 | 217 | 1.25 | 0.60 | 48 | 1 | 4 | 0.13 | 0.06 |
| Stranger approaching owner outside | cb16 | 217 | 1.18 | 0.50 | 42 | 1 | 4 | 0.57 | 0.09 |
| Family member approaching eating dog | cb17 | 215 | 1.46 | 1.01 | 69 | 1 | 5 | 0.94 | na* |
| Mailman visiting | cb18 | 215 | 1.52 | 0.82 | 54 | 1 | 5 | 0.03 | 0.06 |
| Family member removing dog's food | cb19 | 207 | 1.40 | 1.05 | 75 | 1 | 5 | 0.95 | na* |
| Stranger passing house with dog in yard | cb20 | 213 | 1.59 | 0.82 | 52 | 1 | 4 | 0.00 | na* |
| Stranger trying to touch dog | cb21 | 217 | 1.30 | 0.72 | 55 | 1 | 5 | 0.99 | na* |
| Cyclist passing house with dog in yard | cb22 | 213 | 1.40 | 0.73 | 52 | 1 | 4 | 0.00 | na* |
| Unknown male dog approaching leashed dog | cb23 | 216 | 1.90 | 1.16 | 61 | 1 | 5 | 0.23 | 0.14 |
| Unknown female dog approaching leashed dog | cb24 | 216 | 1.59 | 1.08 | 68 | 1 | 5 | 0.16 | 0.10 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Family member staring at dog | cb25 | 216 | 1.07 | 0.35 | 33 | 1 | 4 | 1.00 | na* |
| Unknown dog visiting dog's home | cb26 | 204 | 1.62 | 1.00 | 62 | 1 | 5 | 0.15 | 0.09 |
| Cat entering garden | cb27 | 213 | 2.00 | 1.11 | 56 | 1 | 5 | 0.00 | na* |
| Stranger visiting dog's home | cb28 | 217 | 1.21 | 0.51 | 42 | 1 | 4 | 0.44 | 0.05 |
| Unknown dog barking/attacking dog | cb29 | 216 | 2.28 | 1.29 | 57 | 1 | 5 | 0.23 | 0.16 |
| Family member stepping over dog | cb30 | 217 | 1.08 | 0.41 | 38 | 1 | 4 | 1.00 | na* |
| Family member taking items stolen by dog | cb31 | 201 | 1.40 | 1.04 | 74 | 1 | 5 | 0.04 | 0.12 |
| Aggression towards other family dogs | cb32 | 117 | 1.35 | 0.82 | 61 | 1 | 5 | 1.00 | na* |
| Familiar dog approach. dog at favourite place | cb33 | 119 | 1.19 | 0.56 | 47 | 1 | 4 | 1.00 | na* |
| Familiar dog approaching eating dog | cb34 | 119 | 1.92 | 1.26 | 66 | 1 | 5 | 0.71 | 0.39 |
| Familiar dog approach. dog with favourite toy | cb35 | 118 | 1.85 | 1.11 | 60 | 1 | 5 | 0.26 | 0.24 |
| *Shortened CBARQ scores:* | | | | | | | | | |
| Aggression towards strange humans | scb5 | 241 | 0.47 | 0.47 | 100 | 0.13 | 1.66 | 0.90 | na* |
| Aggression towards owners | scb6 | 241 | 0.50 | 0.53 | 106 | 0.12 | 1.57 | 0.88 | na* |
| Aggression towards other dogs | scb7 | 241 | 1.09 | 0.93 | 85 | 0.29 | 3.19 | 0.31 | 0.10 |
| *CBARQ factors:* | | | | | | | | | |
| Stranger-directed aggression | stragg | 264 | 0.42 | 0.56 | 132 | 0.01 | 3.41 | 0.87 | na* |
| Owner-directed aggression | ownagg | 264 | 0.40 | 0.61 | 153 | 0.01 | 2.89 | 0.82 | na* |
| Dog-directed aggression | dogagg | 264 | 0.94 | 0.98 | 105 | 0.01 | 4.01 | 0.43 | na* |
| Familiar dog-directed aggression | fdgagg | 115 | 0.65 | 0.86 | 133 | 0.01 | 4.01 | 0.45 | na* |

+ One was added to the original CBARQ items and shortened CBARQ scores for technical reasons. As a result of this, means and minimum and maximum values are higher than the values shown in section 2.2.
* Standard errors for estimates could not be obtained.

available for most dogs (Table 1).

Finally, we analysed a fourth type of behavioural measure: CBARQ factors. In these factors, CBARQ scores and shortened CBARQ scores were combined so that original CBARQ scores were used when available, and regressed shortened CBARQ scores were used as an approximation of CBARQ scores for the deceased 83 dogs of which we had no CBARQ scores available. The CBARQ factors included familiar dog directed aggression in addition to the three types of aggression mentioned above. These factors were available for all dogs in the study except for familiar dog-directed aggression that was only available for dogs living in a household with more than one dog (Table 1).

**Statistical analyses**

Variance components needed for obtaining heritability estimates for the aggression measures were estimated with the restricted maximum likelihood (REML) method (Patterson and Thompson 1971), using univariate analyses and an animal model with the VCE4.2.4 software (Groeneveld 1997; VCE4 User's guide and reference manual, version 1.0. URL= http://w3.tzv.fal.de/~eg/vce4/manual/manual.html ; link accessed 12-2005). REML procedures are commonly used in quantitative genetics as they provide a powerful approach for analysing unbalanced data designs with complex known pedigrees (for more information on REML and variance component estimation, see e.g. Lynch and Walsh 1998). Best linear unbiased prediction (BLUP) breeding values were estimated for the dogs for owner impressions for human- and dog-directed aggression using PEST software (Groeneveld 1994; PEST user's manual; URL= ftp://ftp.zgr.fal.de/pub/pest/doc/manual.ps.june94 ; link accessed 12-2005). Pearson product moment correlation coefficients were calculated between these two breeding values to get an impression on to what extent these two traits are related to each other genetically. Estimating genetic correlations directly between the traits was not reasonable due to the small data size.

The following linear animal model was assumed in the analyses for the owner impression traits:

$$y_{ijk} = \mu + sex_i + age_j + a_k + e_k$$

where $y_{ijk}$ is the observed value for owner impression score for animal $k$, $\mu$ is the general mean in the population, $sex_i$ is the fixed effect of the reproductive status $i$ ($i$=1 to 4, with 1=intact male, 2=castrated male, 3=intact female, and

4=castrated female), $age_j$ is the fixed effect of the age $j$ ($j$=1 to 11, with 1=0.5 to 1 year old, 2=1 to 2 years old, …, 10=9 to 10 years old, and 11=over 10 years old), $a_k$ is the random additive genetic effect (i.e. breeding value) of the animal $k$, and $e_k$ is the random residual effect related to the animal $k$. The age and reproductive status of the dogs had been recorded at the same time as the owner impressions.

For the other behavioural measures a similar linear animal model was used, but no fixed effects were included in the model. Some of the fixed effects considered in the mixed model included reproductive status and age, the origin of the dog (e.g., shelter, breeder, private), and various effects related to the relationship of the owner with the dog (e.g., participation in obedience courses; ways to react to misbehaviour of the dog). The majority of the fixed effects did not have any effect on the genetic parameter estimates of the aggression measures and were therefore excluded from the final model. This does not necessarily mean that these effects do not influence canine aggression, but is rather the result of a poor distribution of dogs over most fixed effect classes. In addition, inclusion of fixed effects into the model caused serious overfitting problems due to the small size of the data set (115–264 observations depending on the measure in question). Consequently, in this paper we present for the CBARQ traits only results estimated with a model that did not include any fixed effects in addition to the general mean.

In all analyses, distributions of animal effects and residuals were assumed to be multivariate normal with zero means and with $Var(a) = A\sigma_a^2$ and $Var(e)=I\sigma_e^2$, where A is the numerator relationship matrix and I is an identity matrix. All random effects were assumed to be mutually independent.

## Results

The means, standard deviations, coefficients of variation and minimum and maximum values of all studied aggression measures are presented in Table 1. The distributions of observations were skewed for most of the aggression measures so that the majority of dogs classified in the lower (less aggressive) classes and only a few dogs had been classified in the most extreme (aggressive) classes. This was especially true for the original CBARQ items. Consequently, the means of the aggression measures were low and standard deviations fairly high, resulting in very high coefficients of variation for most measures. The owner impressions had the best distributions with a substantial number of dogs classified in the more aggressive classes. The distributions of the shortened CBARQ scores and the CBARQ factors were quite flat, which

was probably due to the way these measures were formed by regressing from the original CBARQ items.

The effect of reproductive status on owner impressions for human-directed aggression was moderate, with a difference of 0.72 scores between the two extreme reproductive status classes (Figure 3). Castrated males had the highest scores for human-directed aggression, i.e. they were evaluated as the most aggressive. Reproductive status had a much smaller effect on dog-directed aggression, with a difference of 0.14 scores between the two extreme reproductive status classes. Again, castrated males had been evaluated as the most aggressive, while intact males were evaluated even slightly less aggressive than castrated and intact females.

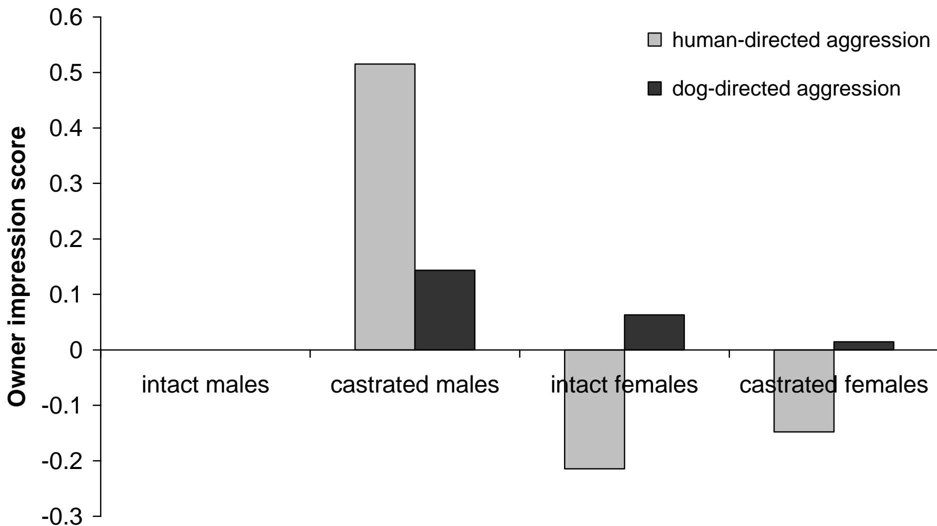



**Figure 3.** Effect of reproductive status on owner impressions on human- and dog-directed aggression, expressed relative to scores for intact males.

The effect of age on owner impressions for human-directed aggression was considerably higher than the effect of reproductive status (Figure 4). The difference between the two extreme age classes was 1.32 scores. The owners evaluated the youngest dogs as the most aggressive to humans. From age class 5 onwards there was almost no difference between the age classes for owner impression on human-directed aggression. For dog-directed aggression there were no clear differences between the age classes. The effect of age was smaller in whole with a difference of 0.59 points between the two extreme age classes.

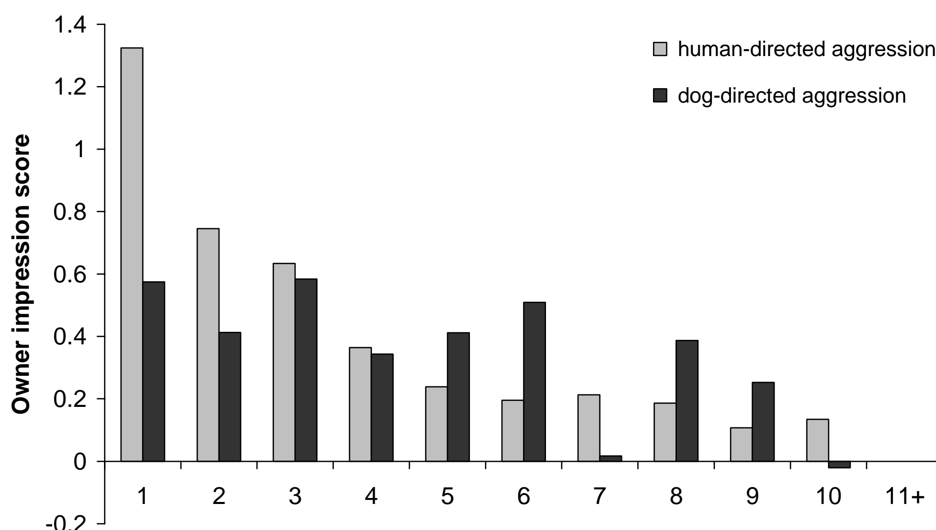



**Figure 4.** Effect of age on owner impressions on human- and dog-directed aggression, expressed relative to scores for dogs over 10 years of age.

The heritability estimates for the aggression measures varied for the full possible range between 0 and 1 (Table 1). There were five measures (all CBARQ items) that had heritability estimates of 1.00. In addition, eleven measures (two owner impressions, five CBARQ items, two shortened CBARQ scores and two CBARQ factors) had heritability estimates over 0.75. Unfortunately, standard errors could not be obtained for the high heritability estimates of CBARQ items, shortened CBARQ scores and CBARQ factors. Consequently their reliability is somewhat questionable, or at least it cannot be assessed properly. The most reliable heritability estimates were obtained for the owner impressions on human- and dog-directed aggression. Both estimates were very high, 0.77 and 0.81 respectively, and they had fairly low standard errors (0.09).

The Pearson product moment correlation coefficient between the BLUP breeding values for owner impressions on human- and dog-directed aggression was 0.40. Although the Pearson correlation underestimates the true genetic correlation due to inaccuracies in estimating the breeding values, this rather low value suggests that human- and dog-directed aggressions are genetically different although partially related traits. This relationship between the traits is also demonstrated in Figure 5, where the estimated breeding values for human- and dog-directed aggression are plotted against each other.

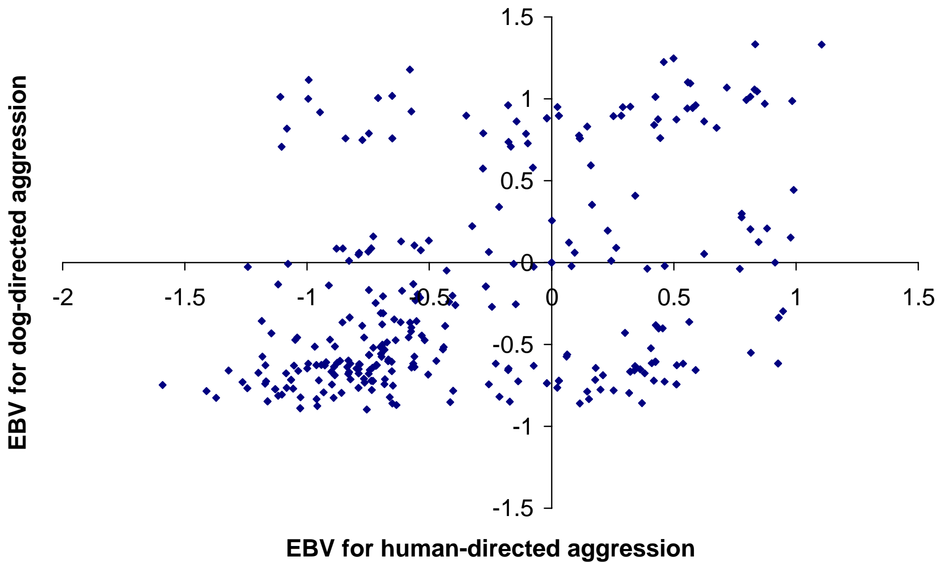



**Figure 5.** Relationship between the estimated breeding values (EBV) for owner impressions on human-directed aggression and dog-directed aggression.

# Discussion

The high aggression scores of castrated males relative to intact males in this study most likely result from the common practice of castrating aggressive dogs in the hope that they will become less aggressive. The results suggest that aggressive behaviour influences castration decisions more in males than in females, as there was little difference in aggression scores between castrated and intact females. We estimated the effect of reproductive status with a mixed model, so it is corrected for the effects of the age of the dog and the genetic effects. The estimated castration effect suggests that, even after castration, the owners judged male dogs that were castrated due to aggression as more aggressive than dogs that had not been considered aggressive enough to warrant castration. Neilson *et al.* (1997) also reported that castration is only effective in decreasing aggressive behaviour in less than a third of dogs. Similar observations on the relationship between the reproductive status and aggressive behaviour have been made for instance by Podberscek and Serpell (1996) in English Cocker Spaniels and Reisner *et al.* (2005) in English Springer Spaniels. In the study of Podberscek and Serpell, the significant positive

association between neutering and aggression largely disappeared when dogs neutered specifically because they had been aggressive were removed from the analysis. Reisner *et al.* (2005) observed that castration was often the result of aggressive behaviour rather than a contributing cause, especially in male dogs.

The effect of age on the aggression scores in this study, with dogs under 2 years being the most aggressive, is partially related to the method of recruiting dogs rather than a pure age-effect. Owners usually encountered aggression problems and entered the study when their dog was approaching social maturity at 1.5 years of age, while the non-aggressive relatives were often recruited when they were older. However, similar onset of problem behaviour was also observed by Lund *et al.* (1995) in their study of behavioural problems including several forms of aggression in different dog breeds in Denmark. In addition, Guy et al. (2001) reported the highest frequency of biting behaviour towards owners in dogs less than one year of age in their study on Canadian dog populations. On the other hand, in the study of Reisner et al. (2005) dogs older than 3 years were significantly more aggressive towards their owners than younger dogs, but they specifically attempted to study aggressive behaviour past the age of social maturity.

At first glance the very high heritabilities obtained in this study for many of the aggression measures seem to point to a fairly simple genetic background for at least human-directed aggression in the studied population of Golden Retrievers. However, the results should be approached with some caution because the data size is very small for variance component estimation, even with the inclusion of the pedigree structure. The poor data structure with unbalanced distributions of most measures further adds to the problem, since the REML method assumes normally distributed traits, although it is also quite robust against distribution violations. At most, we feel that the heritability estimates suggest that for instance cb10 (CBARQ item about aggression when a strange adult approaches the leashed dog) reveals more genetic differences in aggression between the dogs than cb9 (CBARQ item about aggression when verbally corrected by the owner). Their absolute values should not be taken as being the true value.

Based on the heritability estimates, the best behavioural measures for further genetic studies seem to be the owner impressions on human- and dog-directed aggression. The distributions of the owner impression traits were the best suited for variance component estimation using the REML method. The number of observations was also the highest for these two measures, and they could be fitted with proper mixed animal models. Consequently, these heritability estimates are more reliable than the estimates for any of the

CBARQ derived measures. A practical advantage of using these measures for phenotyping is that it is relatively easy and cheap to obtain owner impressions.

The good performance of the owner impressions relative to the more objective CBARQ based measures is somewhat surprising. Usually owners are not considered skilled enough in observing behaviour of their dogs, so that using their impressions might lead to biased results (Galac and Knol 1997; Hart 1995; van der Borg *et al.* 1991). In addition, information provided by owners is likely to reveal only a limited number of phenotypic classes (such as "aggressive" and "non-aggressive"), while a more detailed classification might be needed for genetic studies. However, for this data set, classifying the dogs in only three classes seemed to be sufficient. The "generalness" of the owner impressions might have actually gotten more information out of the very small data set than the more detailed classification of the CBARQ related measures. Aggressive behaviour is also very salient for owners, so it might be relatively easy to evaluate.

Other potential candidate measures for further studies could be some of the original CBARQ items with very high heritability estimates (e.g., cb10, cb11, cb16 and cb21 that are related to stranger-directed aggression, and cb14, cb17, cb19, cb25 and cb30 that are related to owner-directed aggression; Table 1). The shortened CBARQ scores about stranger- and owner-directed aggression and the CBARQ factors for stranger- and owner-directed aggression can also be considered because they had similarly high heritability estimates. In fact, the measures cb10, cb11, cb16 and cb21 are included in the factor for stranger-directed aggression, and the measures cb14, cb17, cb19, cb25 and cb30 are included in the factor for owner-directed aggression (section 2.2), which may explain their similar heritability estimates. Although the standard errors of these very high heritability estimates could not be obtained, they seem to point to clear genetic differences between the dogs with respect to human-directed aggression. For studying dog-directed aggression the CBARQ derived measures are not that good because none of them (CBARQ items, shortened scores or factors) seems to be able to pick up the genetic differences between the dogs as well as the owner impression.

Using the original CBARQ items for genetic studies is not totally straightforward because they describe only one specialised expression of aggressive behaviour at a time. Consequently, if CBARQ items were to be used for further genetic studies on human-directed aggression in this data set, it might be more recommendable to use the shortened CBARQ scores or the CBARQ factors for stranger- and owner-directed aggression. However, using these combined measures for genetic studies can be problematic as well: they might mask the "true" genetic background of the studied traits because they

combine information from many different sources that can be partially contradictory. If a larger data set could be obtained, it would be very interesting to investigate whether the genetic and phenotypic correlations between the original CBARQ items match the structure of the factors. Despite the problems, in long term the CBARQ-derived measures seem very interesting for genetic studies of dog behaviour.

Conclusions that can be drawn from the present study are limited for two reasons. First, and perhaps most importantly, a larger data set is needed for reliable quantitative genetic analyses. Such data set should include at least 500 to 1000 dogs with own phenotypes. From this size of data it is already possible to study the genetic effects of "normal" behavioural traits that have a much smaller genetic component than Golden Retriever aggression. Examples from literature include hunting behaviour in Finnish Spitz (Karjalainen *et al.* 1996), Finnish Hound (Liinamo *et al.* 1997), German Short- and Wire-haired Pointers and Breton (Brenøe *et al.* 2002) and Flat-coated Retriever (Lindberg *et al.* 2004), and other working behaviour traits in German Shepherd and Labrador Retriever (Wilsson and Sundgren 1997; Ruefenacht *et al.* 2002; Strandberg *et al.* 2005).

Second, for quantitative genetic studies one should probably try to avoid selective sampling of dogs like we did in this study. We only collected information from aggressive dogs and their close relatives, but we did not sample all descendants of the sires and dams. This would require more cooperation of breeders, which is often difficult to achieve. For family-based molecular genetic studies, it is not necessary to sample such a large number of progeny. However, the selective sampling might bias the results of quantitative genetic studies. Before any generalisations can be made, the results need to be confirmed with more research on a larger data set and in different breeds.

## Conclusion

The owner impressions on human- and dog-directed aggression seem to be the best candidate measures for further genetic studies. They had very high heritability estimates, low standard errors, and the best data quality. Human- and dog-directed aggression seem to be genetically different, but partially related traits. They should therefore be considered separately in further molecular genetic studies.

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CHAPTER 3

# Molecular genetic analysis

# 3.1

# Isolation and characterization of the canine serotonin receptor 1A gene (*htr1A*)

Linda van den Berg, Serge A. Versteeg, and Bernard A. van Oost

This section has been adapted from "Isolation and characterization of the canine serotonin receptor 1A gene (*htr1A*)" *Journal of Heredity* 2003; **94**(I):49-56.

## Abstract

Although the serotonergic system and *htr1A* have been studied extensively, little is known about the canine serotonin receptor 1A. We are interested in this receptor in the dog because it is likely to be involved in behavioural disorders such as anxiety. Therefore, we isolated a canine BAC clone containing *htr1A*. We used this clone to determine the complete coding sequence of the canine gene. Radiation hybrid (RH) mapping showed that *htr1A* is part of a conserved linkage group that also includes the survival of motor neuron 1 (*smn1*) gene. *Htr1A* is estimated to be located about 7.3 Mb from *smn1* on CFA02. In addition, we report a possible breed-specific variant of the gene in four Golden Retrievers.

## Introduction

Having survived more than 750 million years of evolution, serotonin (5-hydroxytryptamine, 5-HT) is expected to be of great importance in many living organisms (Peroutka 1995). 5-HT functions as a hormone, a mitogen and a neurotransmitter. Its significance is not only evident from its evolutionary age, but also demonstrated by the fact that it is used by one of the most extensive signalling systems in the brain. From the raphe nuclei of the brain stem, serotonergic neurons project widely throughout the central nervous system. Scientists have identified at least 14 receptors that mediate its biological effects (Hoyer *et al.* 1994). There has always been much interest in the serotonergic system, because it plays a role in central nervous system processes regulating fear, anxiety, aggression, sleep, and feeding behaviours (Lucki 1998).

One of the 14 serotonin receptors known to date is serotonin receptor 1A. Its activity is mediated by G-proteins that inhibit adenylate cyclase activity. It can be found as an autoreceptor on serotonergic cell bodies and dendrites in the raphe nuclei. In addition, it is located on postsynaptic targets of serotonin release in a number of limbic structures (see Barnes and Sharp 1999 for a review). Stimulation of $5\text{-HT}_{1A}$ autoreceptors inhibits cell firing and serotonin release. This enables the receptor to modulate the activity of the serotonergic system (Corley *et al.* 1992; Jolas *et al.* 1995; Sprouse and Aghajanian 1987). Although results are conflicting (Olivier *et al.* 2001; Pattij 2002), the $5\text{-HT}_{1A}$ receptor has been associated with anxiety, depression, aggression and stress response. In humans and animals, (partial) $5\text{-HT}_{1A}$ receptor agonists have been reported to have anxiolytic effects (Cervo *et al.* 2000; DeVry 1995; File *et al.* 1996; Oshima 2001). Knockout mice lacking the gene encoding serotonin receptor 1A show heightened anxiety and stress response and an antidepressant-like phenotype (Heisler *et al.* 1998; Parks *et al.* 1998; Ramboz *et al.* 1998).

Kobilka *et al.* (1987) cloned and mapped the human gene encoding serotonin receptor 1A (*HTR1A*). Currently, the complete coding sequence of *htr1A* is known for several organisms, including human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), *Caenorhabditis elegans* and *Drosophila melanogaster*. The *htr1A* gene is intronless, has seven predicted transmembrane-spanning domains, and it contains sites for glycosylation and phosphorylation. It is localised on the human chromosome 5q11.2-q13.

This study on canine *htr1A* was performed in the context of a research project involving canine fear and aggression. Extreme levels of fear and aggression in dogs can result in biting incidents. These have serious implications for the victim and for the dog. Several owners consider euthanasia for their pet in such cases (Galac and Knol 1997). In order to reveal the

aetiology of these behavioural problems, we are studying parts of the canine serotonergic system. Although an enormous number of studies have been performed on the serotonergic system in general and the 5-$HT_{1A}$ receptor in particular, little is known about the canine serotonin receptor 1A. In this section we describe the isolation of a canine bacterial artificial chromosome (BAC) clone containing *htr1A,* the complete coding sequence of this gene, and analysis of its position on the physical map of the dog. In addition, we report a possible breed-specific variant of the gene in four Golden Retrievers.

## Materials and methods

### Animals and DNA isolation

Human and Golden Retriever genomic DNA was isolated from whole blood leucocytes by the salt extraction method of Miller *et al.* (1988). Great Dane genomic DNA was extracted from spleen. The four Golden Retrievers used in this study were privately owned, unrelated dogs. The Retrievers visited our clinic in the context of a research project involving canine fear and aggression. Their owners did not consider the dogs to be either aggressive or anxious.

### BAC library screening

The coding sequence of human *HTR1A* (GenBank Accession Number: X57829) was used to design primers 1 and 2 (Table 1). These primers amplified a 635 bp fragment in a PCR using 200 ng human genomic DNA, 0.5 µM primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 units *Taq* DNA polymerase in 1x Gibco-BRL buffer in a 100 µl reaction volume. This 635 bp probe (*htr1A*-635) was labelled with [$\alpha$-$^{32}$P] dATP with a megaprime DNA labelling kit (Amersham, Piscataway, NY).

The canine genomic BAC library RPCI-81, derived from a Dobermann (Li *et al.* 1999) was screened with probe *htr1A*-635 as previously described (van de Sluis *et al.* 1999). This resulted in only one positive colony: BAC 160O12. For further applications, the alkaline lysis method as described on the BacPac website (http://www.chori.org/bacpac/) was used for BAC DNA isolation.

### Southern blot

To confirm the identity of the BAC clone, genomic Golden Retriever DNA, genomic Great Dane DNA and BAC clone 160O12 DNA were digested with

**Table 1.** Primer pairs used in this study ($T_A$ = annealing temperature).

| Primer numbers | Sequence (5´ – 3´) | Position [a] | Length of product (bp) | $T_A$ (°C) |
|---|---|---|---|---|
| 1 | GAC TAC GTG AAC AAG AGG AC | 427-446 [b] | 635 | 60 |
| 2 | AAG GTG CCC ATG ATG ATG C | 1043-1061 [b] | | |
| 3 | GGC AAC AAC ACC ACC TCG TC | 25-44 | 417 | 56 |
| 4 | CTT GTT CAC GTA GTC GAT GG | 422-441 | | |
| 5 | GAC TAC GTG AAC AAG AGG AC | 427-446 | 461 | 56 |
| 6 | TGC ACT TCA ATC ACC TCC AG | 868-887 | | |
| 7 | CCT GGA GGT GAT TGA AGT GC | 867-886 | 389 | 54 |
| 8 | ACT TGC ACC TGA CGA TCT TC | 1236-1255 | | |
| 9 | GCA GGC ATG GAG GGG CTC AG | -6-14 | 447 | 56 |
| 4 | CTT GTT CAC GTA GTC GAT GG | 422-441 | | |
| 10 | GGC GCT ACA ACC TCA ATT TTC | 503-523 [c] | 265 | 55 |
| 11 | TTG GAT GTA AAA CAG AAA ACA TCA T | 743-767 [c] | | |
| 12 | GTG GAA AGT TGG TGA CAA ATG | 258-278 [d] | 202 | 55 |
| 13 | CTC CTG AGT ATC CTG TTC TAC | 439-459 [d] | | |
| 14 | GGG ACG CTC GGC AAC GCT ACT GG | 58-80 | 716 | 67 |
| 15 | GGC TCG CCG TTC ACG CTC TTC CTG | 750-773 | | |
| 16 | CCT TTG GCG CTT TCT ACA TCC | 599-619 | 707 | 60 |
| 17 | ACC GGG CGG GCC TTC TCG TC | 1286-1305 | | |

a Unless stated otherwise, all positions are based on the canine *htr1A* sequence published in this section.
b The position of this primer is based on the human instead of canine *htr1A* sequence (accession number X57829).
c The positions of primers 10 and 11 are based on the human *CRTL1* sequence (accession number NM_001884).
d The positions of primers 12 and 13 are based on the canine *smn1* sequence (accession number U50746)

*Eco*RI. The resulting fragments were separated on a 0.7% agarose gel, transferred to Hybond $N^+$ filter (Amersham) and hybridised at 65°C with the *htr1A*-635 probe.

## Sequence analysis of the canine *htr1A*

We determined the major part of the canine *htr1A* sequence by means of sequencing subcloned BAC 160O12 fragments. BAC 160O12 DNA was

digested with *Sau*3AI. The resulting fragments were ligated into *Bam*HI digested pZeRO$^{tm}$-1 vector with T4 DNA ligase, transformed into TOP10F' bacterial cells, and plated on low-salt LB plates with 50 µg/ml zeocin. We transferred zeocin-resistant colonies to Hybond N$^{+}$ filters that were hybridised with the *htr1A*-635 probe. Positive colonies were picked and grown overnight. We isolated DNA from these cultures through miniprepping with a Promega DNA purification system. The subcloned DNA was sequenced with T7 (5´-TAA TAC GAC TCA CTA TAG GG –3´) and SP6 (5´- ATT TAG GTG ACA CTA TAG –3´) primers using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) with BigDye Termination Mix.

The first 50 bp of the gene were sequenced by means of direct BAC DNA sequencing. Twenty pmol of a reverse primer (5´- GAG GTG ATC ACT TGG TAG CTG - 3´) was used in a 30 µl tercycle reaction with 4 mM MgCl2, 12 µl Big Dye Terminator Ready Reaction Mix and 750 ng BAC DNA. The tercycle consisted of 5 min at 95ºC, followed by 34 cycles of 30 s at 95ºC, 10 s at 56ºC, and 4 min at 60ºC. We purified tercycle products using multiscreen 96-well filtration plates (Millipore) and sequenced them in 10 µl distilled water with an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Three primer pairs (3 and 4, 5 and 6, 7 and 8; see Table 1) were designed based on the preliminary canine coding sequence. These primers amplified adjacent fragments in PCRs using BAC clone 160O12 DNA as a template. The PCR products were sequenced several times. We checked the first and last 50 bp of the exon by direct BAC DNA sequencing. In this way, every base in the preliminary sequence was checked at least once.

We calculated homologies with human and murine sequences with MEGALIGN (LASERGENE Software). Sequences retrieved have the following GenBank Accession Numbers: X57829, AB041403, XM_003692, AF498978 (human), U39391 S67168, XM_122644, and NM_008308 (mouse). We derived predicted positions of the transmembrane regions in the protein from the SWISSPROT website (http://srs6.ebi.ac.uk/srs6bin/cgi-bin/wgetz?-newId+-e+[libs%3d{SWALL_SP_REMTREMBL}-acc:P08908]).

## Radiation hybrid mapping

The canine chromosome segments corresponding to HSA05 were studied in order to select two genes flanking *HTR1A* in the human genome: cartilage linking protein 1 (*CRTL1*) and survival of motor neuron 1, telomeric (*SMN1*) (Figure 1). The commercially available canine whole genome radiation hybrid (RH) panel T72 (3000 RAD, Research Genetics, http://www.resgen.com/

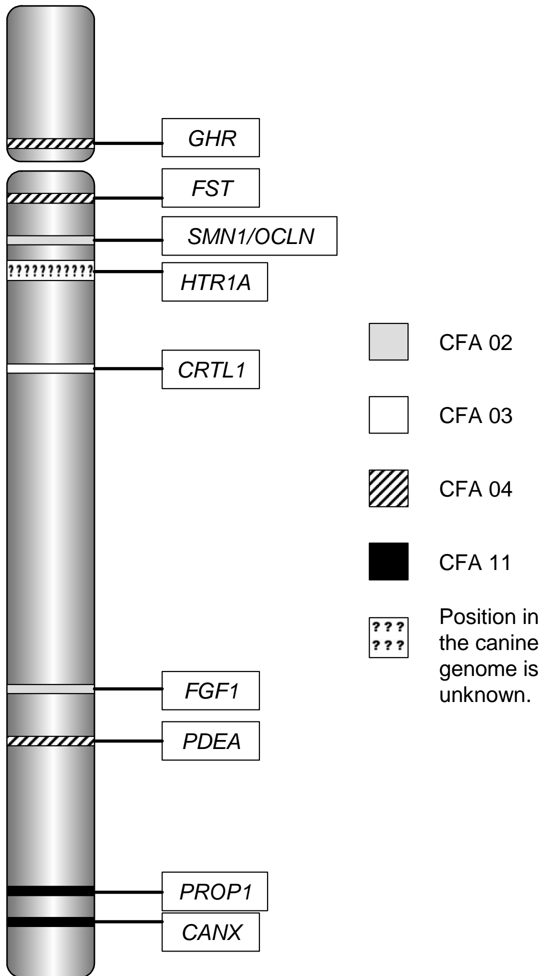


**Figure 1.** Schematic representation of human chromosome 5. The bands mark genes that were mapped in human as well as dog. The position of the bands along the chromosome is based on genomic contigs of HSA 05 on the Human Map Viewer website (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/map_search; accessed in October 2002) The pattern of a band represents the position of the gene in the canine genome (source: http://www-recomgen.univ-rennes1.fr/cgi-dog/display-hsa.prog?hsa=5; accessed in October 2002). The position of *htr1A* on the canine genome was not yet known when we started this study. *GHR*=growth hormone receptor; *FST*= follistatin; *SMN1*=survival of motor neuron 1, telomeric; *OCLN* =occludin; *CRTL1*= cartilage linking protein 1; *FGF1*=fibroblast growth factor 1 (acidic); *PDEA*= phophodiesterase 6A, cGMP-specific, rod, alpha; *PROP1*=prophet of Pit1, paired-like homeo domain transcription factor; *CANX*=calnexin.

products/CRH.php3) was used to determine linkage between *htr1A* and both of these genes. Primers 9 and 4 were used to establish retention of *htr1A* in the hybrids. We used primers 10 and 11 for *crtl1* and 12 and 13 for *smn1* (Table 1).

PCR reactions contained 25 ng DNA, 0.53 µM primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.6 U Platinum Taq Polymerase (Invitrogen), and 1x Gibco-BRL buffer in a total reaction volume of 15 µl. We used the following PCR program: 3 min 94ºC initial denaturation, followed by 35 cycles of 30 s at 94ºC, 30 s at $T_A$ and 30 s at 72ºC. Final step was 4 min at 72ºC. The primers were first tested on genomic canine, hamster and 2:1 hamster/canine DNA in order to confirm their specificity for dog DNA. A two-point LOD score was

computed for both *htr1A/smn1* and *htr1A/crtl1* with RH2PT software (RHMAP, version 3.0; Boehnke *et al.* 1991).

### Partial sequence analysis of *htr1A* in four Golden Retrievers

Analysis of *htr1A* sequences in genomic DNA of four Golden Retrievers was performed by means of PCR product sequencing, using primers 14 and 15 and 16 and 17. We obtained reliable sequencing results for base pairs 115-1250 in each of these dogs.

## Results

### BAC library screening and Southern blot

BAC library screening resulted in one positive BAC clone: 160O12. Both this clone and genomic dog DNA showed a single 9.4 kb band in a Southern blot analysis using a 635 bp human probe (Figure 2). We considered it unnecessary to repeat the Southern blotting procedure with a full-length probe because the *htr1A* exon contains no *Eco*RI sites. These results imply that we have isolated a genomic clone of canine *htr1A* and that the *htr1A* gene is single-copy in the canine genome.

### Sequence analysis

With the help of the 160O12 BAC clone, we managed to reveal the entire coding sequence of *htr1A* in the domestic dog, including 22 bp at the 5´-flanking side and 45 bp at the 3´-flanking side. The gene consists of only one exon, with

**Figure 2.** Southern blotting results. Genomic Golden Retriever DNA ► (lane a), genomic Great Dane DNA (data not shown, but similar) and BAC 160O12 DNA (lane b) were digested with *Eco*RI, separated on a 0.7% agarose gel, transferred to Hybond $N^+$ filter and hybridised at 65°C with the *htr1A*-635 probe.

an open reading frame of 1272 bp (Figure 3). In dogs, the gene is longer than in humans (1269 bp) and mice (1266 bp). Homology of the exonic nucleotide sequence with human and murine sequences is high (89% and 85% sequence identity, respectively). The corresponding protein consists of 423 amino acids. Amino acid homology reached 92% with human and 85% with mouse.

```
CCCCCCCGCCGGGCGCGCAGGC

ATG GAG GGG CTC AGC CCC CGA CAG GGC AAC AAC ACC ACC TCG TCC GAG GGG CCC   54
 M   E   G   L   S   P   R   Q   G   N   N   T   T   S   S   E   G   P    18
TTC GGG ACG CGC GGC AAC GCT ACT GGC ATC TCC GAC GTG ACC TTC AGC TAC CAA  108
 F   G   T   R   G   N   A   T   G   I   S   D   V   T   F   S   Y   Q    36
GTG ATC ACC TCC CTG CTG CTG GGC ACG CTC ATT TTC TGC GCG GTG CTG GGC AAT  162
 V   I   T   S   L   L   L   G   T   L   I   F   C   A   V   L   G   N    54
GCG TGC GTG GTG GCC GCC ATC GCC CTG GAG CGC TCC CTG CAG AAT GTG GCC AAC  216
 A   C   V   V   A   A   I   A   L   E   R   S   L   Q   N   V   A   N    72
TAT CTC ATC GGC TCG CTG GCC GTC ACC GAC CTC ATG GTG TCG GTG CTG GTG CTG  270
 Y   L   I   G   S   L   A   V   T   D   L   M   V   S   V   L   V   L    90
CCC ATG GCC GCG CTG TAC CAG GTG CTC AAC AAA TGG ACG CTG GGA CAG GTC ACC  324
 P   M   A   A   L   Y   Q   V   L   N   K   W   T   L   G   Q   V   T   108
TGT GAC CTA TTC ATT GCC CTC GAC GTG CTG TGC TGC ACC TCG TCC ATC CTG CAC  378
 C   D   L   F   I   A   L   D   V   L   C   C   T   S   S   I   L   H   126
CTG TGC GCC ATT GCG CTG GAC AGG TAC TGG GCC ATC ACG GAC CCC ATC GAC TAC  432
 L   C   A   I   A   L   D   R   Y   W   A   I   T   D   P   I   D   Y   144
GTG AAC AAG AGG ACG CCC CGG CGC GCC GCT GCG CTC ATC TCG CTC ACT TGG CTC  486
 V   N   K   R   T   P   R   R   A   A   A   L   I   S   L   T   W   L   162
ATC GGC TTC CTC ATC TCC ATT CCG CCC ATG CTG GGT TGG CGC ACC CCC GAA GAC  540
 I   G   F   L   I   S   I   P   P   M   L   G   W   R   T   P   E   D   180
CGC TCG GAC CCC GAC GCG TGC ACC ATC AGC AAG GAC CAC GGC TAC ACT ATC TAC  594
 R   S   D   P   D   A   C   T   I   S   K   D   H   G   Y   T   I   Y   198
TCC ACC TTT GGC GCT TTC TAC ATC CCG CTG CTG CTC ATG CTG GTC CTC TAC GGG  648
 S   T   F   G   A   F   Y   I   P   L   L   L   M   L   V   L   Y   G   216
CGC ATC TTC CGC GCC GCG CGC TTC CGC ATC CGC AAA ACA GTC AAG AAG GCG GAG  702
 R   I   F   R   A   A   R   F   R   I   R   K   T   V   K   K   A   E   234
AGG AAG GGA GCG GAC GCC CGC TCC GGG GTG TCG CCA GCC CCG CAG CCC AGG AAG  756
 R   K   G   A   D   A   R   S   G   V   S   P   A   P   Q   P   R   K   252
AGC GTG AAC GGC GAG CCG GGG GGC AGA GAA TGG AGG CAG GGT CCG GGG AGC AAG  810
 S   V   N   G   E   P   G   G   R   E   W   R   Q   G   P   G   S  K(Q) 270
GCT GGG GGG CCT CTG TGC ACC AAC GGC GCG GTG AGG CGG GGC GAC GAC GGC GCC  864
 A   G   G   P   L   C   T   N   G   A   V   R   R   G   D   D   G   A   288
GCC CTG GAG GTG ATT GAA GTG CAC CGC GTG GGC AGC TCC AAA GAG CAC CTG CCG  918
 A   L   E   V   I   E   V   H   R   V   G   S   S   K   E   H   L   P   306
CTG CCC TGC GAG GCT GGC GCC ATC CCT TGC GCC CCC GCC TCC TTC GAG AAG AAG  972
 L   P   C   E   A   G   A   I   P   C   A   P   A   S   F   E   K   K   324
AAT GAG CGC AAC GCC GAG GCC AAG CGC AAG ATG GCC CTG GCC CGG GAG AGG AAA 1026
 N   E   R   N   A   E   A   K   R   K   M   A   L   A   R   E   R   K   342
ACG GTG AAG ACG CTG GGC ATC ATC ATG GGC ACG TTC ATC CTC TGC TGG CTG CCC 1080
 T   V   K   T   L   G   I   I   M   G   T   F   I   L   C   W   L   P   360
TTC TTC ATC GTG GCC CTG GTC CTG CCC TTC TGC GAG AGC AGC TGC CAC ATG CCC 1134
 F   F   I   V   A   L   V   L   P   F   C   E   S   S   C   H   M   P   378
ACC CTG CTG GGC GCC ATA ATC AAC TGG CTG GGC TAC TCC AAC TCC CTG CTC AAC 1188
 T   L   L   G   A   I   I   N   W   L   G   Y   S   N   S   L   L   N   396
CCC GTC ATC TAC GCC TAC TTC AAC AAG GAC TTC CAG AAC GCC TTT AAG AAG ATC 1242
 P   V   I   Y   A   Y   F   N   K   D   F   Q   N   A   F   K   K   I   414
GTC AGG TGC AAG TTC TGC CGC CGA CGG TGA                                 1272
 V   R   C   K   F   C   R   R   R                                       423

CGGCGGCGCGGTCGACGAGAAGGCCCGCCCGGTCCTCTCGGGCCC
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**Figure 3.** Nucleotide sequence and deduced amino acid sequence of canine *htr1A*. The sequence has been deposited in the GenBank database under accession number AY134445. A preliminary sequence was obtained by means of sequencing subcloned BAC 160O12 with T7 and SP6 primers. Subsequently, this sequence was checked by means of sequencing BAC DNA-based PCR products. DNA was translated with EditSeq (LASERGENE Software). The adenine at nucleotide position 808 is printed in bold because this was cytosine in four Golden Retrievers that we studied, resulting in glutamine instead of lysine at amino acid position 270.

Several regions in the protein were even more homologous between the species (Figure 4). The seven hydrophobic (transmembrane) regions consist of amino acid residues 37-62, 74-98, 110-132, 153-178, 192-217, 346-367 and 379-403 in humans, mice and dogs (where the initiator ATG codon is designated number 1). Amino acid composition of these seven regions is 100% identical between dog and human. The murine amino acid composition is different at two positions (residues 177 and 379). Two sites for phosphorylation by protein kinase C can be found at amino acid position 147-151 and 227-232 in the human, canine and murine protein. These regions are 100% conserved. Furthermore, three asparagine residues (10, 11 and 24) that were indicated as potential sites for glycosylation by Kobilka *et al.* (1987) can be found at identical positions in human, mouse and dog.

**Radiation hybrid mapping**

*Htr1A* was retained in 26 hybrids (28.5%). The calculated distance between *htr1A* and *smn1* was 34.8 $cR_{3000}$ (LOD score = 9.7). *htr1A* is expected to be localised about 7.3 Mb from *smn1* on canine chromosome 2 because 1 cR is estimated to be 210 kb for this RH panel (van de Sluis *et al.* 2000) and because *smn1* has been mapped to CFA02. These results imply that *htr1A* will be positioned either between microsatellite markers REN154C04 and REN115P05, or between microsatellite marker C02.466 and gene-based marker *fgf1* on the 2001 integrated dog map, assuming that 1 $cR_{5000}$ corresponds to 100 kb (Breen *et al.* 2001; see Figure 5). Not surprisingly, no linkage was found between *htr1A* and *crtl1*, which has been mapped to canine chromosome 3 (LOD score = 0).

**Partial sequence analysis of *htr1A* in four Golden Retrievers**

Sequence analysis of base pairs 115-1250 in four Golden Retrievers revealed that all of these dogs had cytosine instead of adenine at position 808 in their *htr1A* sequence. Amino acid 270 will therefore be glutamine instead of lysine in these dogs (see Figures 3 and 4). This finding might point to breed-specific differences in the structure of serotonin receptor 1A. No other differences were found between the BAC 160O12 sequence and the genomic Golden Retriever sequence.

| Species | | | | | Position |
|---|---|---|---|---|---|
| DOG | M E G L S P R Q G N | N T T S S E G P F G | T R G N A T G I S D | V T F S Y Q V I T S | 40 |
| HUMAN | M D V L S P G Q G N | N T T S P P A P F E | T G G N T T G I S D | V T V S Y Q V I T S | |
| MOUSE | M D M F S L G Q G N | N T T T S L E P F G | T G G N D T G L S N | V T F S Y Q V I T S | |
| DOG | L L L G T L I F C A | V L G N A C V V A A | I A L E R S L Q N V | A N Y L I G S L A V | 80 |
| HUMAN | L L L G T L I F C A | V L G N A C V V A A | I A L E R S L Q N V | A N Y L I G S L A V | |
| MOUSE | L L L G T L I F C A | V L G N A C V V A A | I A L E R S L Q N V | A N Y L I G S L A V | |
| DOG | T D L M V S V L V L | P M A A L Y Q V L N | K W T L G Q V T C D | L F I A L D V L C C | 120 |
| HUMAN | T D L M V S V L V L | P M A A L Y Q V L N | K W T L G Q V T C D | L F I A L D V L C C | |
| MOUSE | T D L M V S V L V L | P M A A L Y Q V L N | K W T L G Q V T C D | L F I A L D V L C C | |
| DOG | T S S I L H L C A I | A L D R Y W A I T D | P I D Y V N K R T P | R R A A A L I S L T | 160 |
| HUMAN | T S S I L H L C A I | A L D R Y W A I T D | P I D Y V N K R T P | R R A A A L I S L T | |
| MOUSE | T S S I L H L C A I | A L D R Y W A I T D | P I D Y V N K R T P | R R A A A L I S L T | |
| DOG | W L I G F L I S I P | P M L G W R T P E D | R S D P D A C T I S | K D H G Y T I Y S T | 200 |
| HUMAN | W L I G F L I S I P | P M L G W R T P E D | R S D P D A C T I S | K D H G Y T I Y S T | |
| MOUSE | W L I G F L I S I P | P M L G W R A P E D | R S N P N E C T I S | K D H G Y T I Y S T | |
| DOG | F G A F Y I P L L L | M L V L Y G R I F R | A A R F R I R K T V | K K A E R K G A D A | 240 |
| HUMAN | F G A F Y I P L L L | M L V L Y G R I F R | A A R F R I R K T V | K K V E K T G A D T | |
| MOUSE | F G A F Y I P L L L | M L V L Y G R I F R | A A R F R I R K T V | K K V E K K G A G T | |
| DOG | R S G V S P A P Q P | R K S V N G E P G G | R E W R Q G P G S K | A G G P L C T N G A | 280 |
| HUMAN | R H G A S P A P Q P | K K S V N G E S G S | R N W R L G V E S K | A G G A L C A N G A | |
| MOUSE | S F G T S S A P P P | K K S L N G Q P G S | G D C R R S A E N R | A V G T P C A N G A | |
| DOG | V R R G D D G A A L | E V I E V H R V G S | S K E H L P L P C E | A G A I P C A P A S | 320 |
| HUMAN | V R Q G D D G A A L | E V I E V H R V G N | S K E H L P L P S E | A G P T P C A P A S | |
| MOUSE | V R Q G E D D A T L | E V I E V H R V G N | S K G D L P L P S E | S G A T S Y V P A C | |
| DOG | F E K K N E R N A E | A K R K M A L A R E | R K T V K T L G I I | M G T F I L C W L P | 360 |
| HUMAN | F E R K N E R N A E | A K R K M A L A R E | R K T V K T L G I I | M G T F I L C W L P | |
| MOUSE | L E R K N E R T A E | A K R K M A L A R E | R K T V K T L G I I | M G T F I L C W L P | |
| DOG | F F I V A L V L P F | C E S S C H M P T L | L G A I I N W L G Y | S N S L L N P V I Y | 400 |
| HUMAN | F F I V A L V L P F | C E S S C H M P T L | L G A I I N W L G Y | S N S L L N P V I Y | |
| MOUSE | F F I V A L V L P F | C E S S C H M P E L | L G A I I N W L G Y | S N S L L N P V I Y | |
| DOG | A Y F N K D F Q N A | F K K I V R C K F C | R R R | | 423 |
| HUMAN | A Y F N K D F Q N A | F K K I I K C K F C | R Q | | 422 |
| MOUSE | A Y F N K D F Q N A | F K K I I K C K F C | R | | 421 |

**Figure 4.** Alignment of the amino acid sequence of the canine serotonin receptor 1A with human (accession number AF498978) and murine (accession number NM_008308) sequences. Amino acids are presented in groups of ten. Residues that are conserved in the three species are shown in grey background. Predicted transmembrane regions according to SWISSPROT (http://us.expasy.org/cgi-bin/niceprot.pl?P08908) are boxed. Residues 1-36 form the amino terminus; residues 37-62 form the first intracellular loop; etc. Amino acid residues 22 and 270 are depicted in a black box because they are polymorphic in the dog. Amino acid number 22 can be either arginine (Arg, R) or leucine (Leu, L) in dogs. (The detection of this polymorphism will be described in section 3.3 of this thesis.) At position 270 there is a lysine (Lys, K)/glutamine (Gln, Q) polymorphism. The following key residues are underlined: 10, 11, and 24: N-linked glycosylation (SWISSPROT); 109 and 187: disulfide bridge (SWISSPROT); 82: interaction with G-protein (Shih *et al.* 1991).

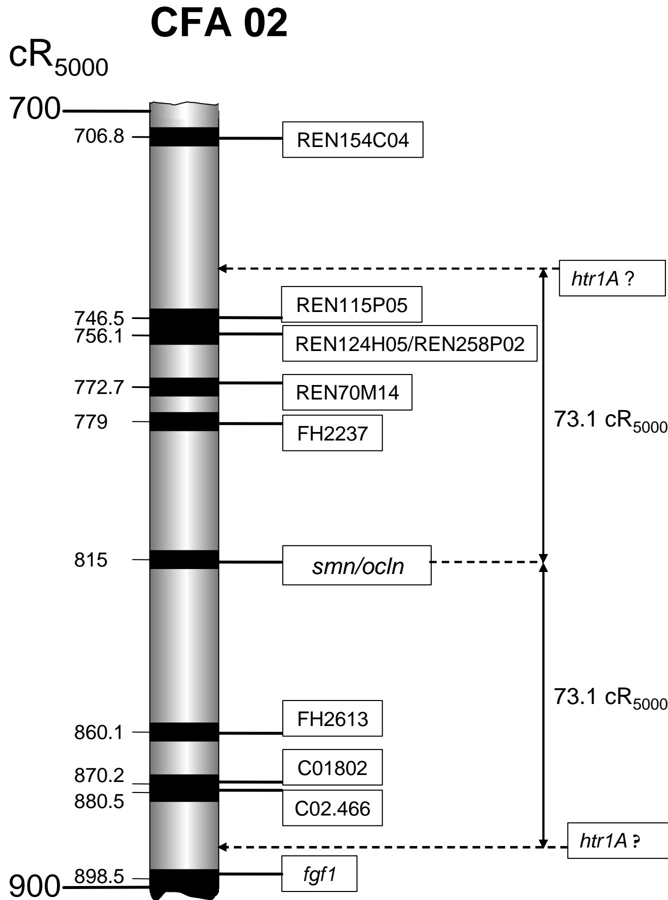


**Figure 5.** Schematic representation of a part of canine chromosome 2, running from 700 $cR_{5000}$ to 900 $cR_{5000}$ based on radiation hybrid data from http://www-recomgen.univ-rennes1.fr/cgi-dog/display-hsa.prog?hsa=5 ; accessed in October 2002. The two possible positions of *htr1A* were calculated on the basis of radiation hybrid mapping of *htr1A* and *smn1* with the help of a commercially available radiation hybrid panel. Markers REN154C04, REN115P05, REN124H05, REN258P02, REN70M14, FH2237, FH2613, C01802, and C02.466 are all microsatellite markers, whereas *smn1* (survival of motor neuron 1, telomeric), *ocln* (occludin), and *fgf1* (fibroblast growth factor 1 (acidic)) are all gene-based markers.

# Discussion

We have isolated a canine BAC clone containing the gene encoding serotonin receptor 1A and we have determined the entire coding sequence of this gene. The 1272 bp intronless gene is very similar to human and murine *htr1A*, especially in transmembrane regions and at the sites for phosphorylation and glycosylation. Canine *htr1A* is more homologous to human *HTR1A* than to murine *htr1A*; 89% of the canine nucleotides are identical to the human sequence, whereas human and mouse share only 85% of their nucleotides. At the amino acid level, dog and human are 92% identical, whereas mouse and man are 88% the same.

Several promoter elements can be recognised in the short stretch of 5´-flanking region that we have sequenced. Human and mouse 5´-flanking regions of *htr1A* have been shown to contain complex TATA-less promoters (Parks and Shenk 1996). It will be very interesting to find out more about the *htr1A* promoter region in the dog by sequencing farther in the 5´ direction.

Radiation hybrid mapping showed that *htr1A* resides on CFA02, at a distance of 34.8 $cR_{3000}$ from *smn1*. These results are in accordance with the human situation, where *HTR1A* and *SMN1* are separated by about 6 Mb on HSA05 (Human Map Viewer, http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/maps.cgi?org=hum&chr =5). Additional detailed mapping will reveal whether *htr1A* is positioned proximally or distally from *smn1*.

An A → C substitution was found in four Golden Retrievers, resulting in a different amino acid at position 270. This amino acid is not situated in a highly conserved region of the protein (Figure 4). The region forms one of the extracellular loops of the receptor. It is not expected that the substitution of residue 270 results in altered ligand-binding properties of the receptor because the ligand binding site of the 5-$HT_{1A}$ receptor is localised in the membrane domains (Shih *et al.* 1991). It will be very interesting to determine the remainder of the *htr1A* sequence in these Golden Retrievers and to compare these sequences with those in other breeds. In addition, a comparison between anxious or aggressive dogs and "normal" dogs of the same breed will be useful.

In conclusion, we have cloned, characterised and mapped one of the genes of the serotonergic system in the dog. These data are valuable for candidate gene studies of behavioural disorders in dogs and they provide a new Type I marker on the Canine Genome Map. Future studies will be directed at determination of the *htr1A* sequence and its promoter region sequence in various dog breeds and in dogs with behavioural disorders.

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# 3.2

# Isolation and characterization of the canine serotonin receptor 1B gene (*htr1B*)

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## Abstract

The serotonin receptor 1B gene (*htr1B*) has been suggested to be involved in mental disorders in humans and other species. We have isolated a canine bacterial artificial chromosome (BAC) clone containing *htr1B*, revealed the coding and surrounding DNA sequence of canine *htr1B* and designed primer sets for genomic sequencing of the gene. A mutation scan in 10 dogs revealed five single nucleotide polymorphisms in the *htr1B* coding sequence. A polymorphic microsatellite repeat was identified by random sequencing of subclones of the BAC. We found evidence for at least four haplotypes in six dogs of the same breed. The chromosomal location of the gene was confirmed by fluorescence *in situ* hybridisation and radiation hybrid mapping. This work provides a starting point for mutation scans and association studies on dogs with behavioural problems.

## Introduction

The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) plays a central role in the molecular biology of the mind. Serotonin is involved in the regulation of a variety of behaviours, including sleep, fear, aggression, mood, and food intake (Lesch and Merschdorf 2000; Lucki 1998). Neurons of the serotonergic system project throughout the central nervous system. Serotonin receptors can be found in primitive life forms such as flatworms (Venter *et al.* 1988). To date, fourteen serotonin receptor subtypes have been identified (Hoyer *et al.* 1994).

One of these serotonin receptors is serotonin receptor 1B. Like most serotonin receptors it is a member of the family of G-protein coupled receptors. The protein has seven transmembrane regions and its activity is mediated by G-proteins that inhibit adenylate cyclase (Hamblin *et al.* 1992; Maroteaux *et al.* 1992). 5-$HT_{1B}$ receptors can be found both pre- and postsynaptically on a variety of neurons in several brain areas. They modulate the release of 5-HT and other neurotransmitters (Hartig 1997; Hoyer *et al.* 2002). The gene encoding serotonin receptor 1B (*htr1B*) has no introns. In human 16 polymorphisms have been discovered in its coding and flanking DNA sequence (Jin *et al.* 1992; Hamblin *et al.* 1992, Maroteaux *et al.* 1992; Sanders *et al.* 2002).

A bulk of studies has suggested involvement of *htr1B* in the aetiology of mental disorders (Sanders *et al.* 2002). Multiple linkage scans have identified a schizophrenia susceptibility gene in the human chromosomal region harbouring *HTR1B* (HSA 6q13, see Sanders *et al.* 2002 for a review). Moreover, an association has been suggested between one of the polymorphisms in the human gene (G186C) and alcoholism, suicidality, and obsessive-compulsive disorder (Huang *et al.* 2003; Sanders *et al.* 2002). Knock-out mice lacking *htr1B* show several behavioural changes, including increased aggression (Saudou *et al.* 1994).

The present study on canine *htr1B* was performed in the context of a research project involving canine fear, aggression and impulsivity. Extreme aggressive behaviour in dogs is a major problem for victim, owner and dog (Hunthausen 1997; Rusch *et al.* 2000). We are studying parts of the canine serotonergic system in order to reveal genetic factors involved in the aetiology of this behavioural problem.

Information about canine *htr1B* is currently limited to its position on the canine map (Guyon *et al.* 2003) and partial nucleotide sequence of the coding region (Sgard *et al.* 1996). This section reports on the isolation and characterization of a canine bacterial artificial chromosome (BAC) clone

containing *htr1B*. We describe the complete coding sequence of the canine gene including non-coding flanking regions and five single nucleotide polymorphisms (SNPs), confirmation of its position in the dog genome, and isolation of a nearby microsatellite marker.

## Materials and methods

### Animals and DNA isolation

The Golden Retrievers, Dobermanns and Beagles used in this study were privately owned, apparently not closely related dogs, except for one sibling pair (Golden Retrievers 3031 and 3032). We had no information about their behavioural characteristics. Canine and mouse (BALBc) genomic DNA was isolated from whole blood leucocytes using the salt extraction method (Miller *et al.* 1988). Great Dane genomic DNA was extracted from spleen.

### BAC library screening

Primers 1 and 2 (Table 1) were based on the coding sequence of *htr1B* in the mouse (GenBank Accession Number: M85151). These primers amplified a 997 bp fragment of *htr1B* in a PCR using 500 ng genomic mouse DNA, 0.5 μM primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 units *Taq* DNA polymerase in 1x Gibco-BRL buffer in a 100 μl reaction volume. This 997 bp probe (*htr1B*-997) was labelled with [α-$^{32}$P] dATP using a megaprime DNA labelling kit (Amersham, Piscataway, NY). The nucleotide sequence of *htr1B-997* is 91% identical to the corresponding region in human *HTR1B*, whereas it is only 67% identical to the closely related murine *htr1D*. We thus did not expect the probe to hybridise with other members of the family of serotonin receptor genes.

We screened the canine genomic BAC library RPCI-81, derived from a Dobermann (Li *et al.* 1999), with a pool of different probes including *htr1B*-997. Filters were prehybridised (one hour) and hybridised (overnight) in Church Buffer at 65ºC, washed, and exposed to film at -70ºC (see http://bacpac.chori.org/highdensity.htm for details). Positive colonies were picked, grown overnight in 3 ml Luria-Bertani medium with chloramphenicol (20 μg/ml) and 1 μl of the overnight bacterial culture was spotted onto a Hybond N$^+$ membrane (Amersham). We hybridised these colonies with probe *htr1B*-997 to ensure that they contained *htr1B*. Positive colonies were picked and grown and the alkaline lysis method as described on the BacPac website (http://bacpac.chori.org/bacpacmini.htm) was used for BAC DNA isolation.

**Table 1.** Primer pairs used in this study ($T_A$ = annealing temperature).

| Primer numbers | Sequence (5´ – 3´) | Position[a] | Length of product (bp) | $T_A$ (°C) |
|---|---|---|---|---|
| 1 | CCT GGA AAG TCC TGC TGG | 128-145[b] | 997 | 60 |
| 2 | GCT TGT TTG AAG TCC TCA TTG G | 1103-1124[b] | | |
| 3 | GGC GAG GAG AGA CAT GGA A | -13-6 | 317 | 62 |
| 4 | TCA CCA GGA TGG AGA CGA G | 286-304 | | |
| 5 | CTC ATC ACC TTG GCC ACC AC | 169-188 | 752 | 60 |
| 6 | CTA GCG GCC ATG AGT TTC TTC | 900-920 | | |
| 7 | ACA TCC TCT ACA CCG TGT ACT C | 611-632 | 680 | 57 |
| 8 | GCC AGA AGA CAG AGC CTC A | 1272-1290 | | |
| 9 | CTT CTC AGG CAT CAT TCT CC | not available | 139 | 57 |
| 10 | CGT GGA GCC TGC TTC TT | | | |

[a] Unless stated otherwise, positions are based on the coding sequence of canine *htr1B* published in this section, where the A of the ATG start codon is designated number 1.
[b] The position of this primer is based on the coding sequence of murine *htr1B* (accession number M85151).

## Southern blot

In order to select a BAC clone containing *htr1B*, 10 µg genomic Golden Retriever DNA, 10 µg genomic Great Dane DNA and 500 ng BAC clone DNA were digested with *Eco*RI. In addition, we digested genomic DNA with *Bgl*II, *Bam*HI and *Pst*I. The resulting fragments were separated on a 0.7% agarose gel and transferred to Hybond $N^+$ membrane (Amersham).

We performed prehybridisation (one hour) and hybridisation (overnight) at 65ºC in 0.5 M sodium phosphate buffer (pH 7.4)/7% SDS/0.1% EDTA using probe *htr1B*-997. The blots were washed 3 times for 5 minutes and 1 time for 15 minutes (genomic DNA) or three times for 15 minutes (BAC DNA) with 40 mM sodium phosphate buffer/0.1% SDS at 65ºC and exposed to film for 3 days at -70ºC (genomic DNA) or 1.15 hours at 20ºC (BAC DNA). We selected the BAC clone with the strongest signal (BAC 18L8).

## Sequence analysis of canine *htr1B*

We determined the majority of the canine *htr1B* sequence by sequencing subcloned fragments of BAC 18L8. BAC 18L8 DNA (1 µg) was digested with

*Sau*3AI, the resulting fragments were purified and ligated into *Bam*HI digested pZErO$^{tm}$-1 vector with T4 DNA ligase (New England Biolabs), transformed into TOP10F' bacterial cells and plated on low salt LB plates with 50 µg/ml zeocin. We transferred zeocin-resistent colonies to Hybond N$^{+}$ membranes and we hybrised DNA from these colonies with the *htr1B*-997 probe. DNA was isolated from the positive colonies with a Promega DNA purification system. The DNA was sequenced with T7 (5´- TAA TAC GAC TCA CTA TAG GG –3´) and SP6 (5´- ATT TAG GTG ACA CTA TAG –3´) primers using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The borders of the gene were sequenced by direct BAC DNA sequencing. We used 20 picomoles of a reverse (5´- ATC ACC AGG ATG GAG ACG AGC A - 3´) or a forward (5´- ACG CGC TGC TGG AGA AGA AG - 3´) primer in a 30 µl tercycle reaction with 4 mM $MgCl_2$, 12 µl BigDye Terminator v3.0 Ready Reaction Mix (Applied Biosystems) and 800 ng BAC DNA. The tercycle consisted of 5 min at 95ºC, followed by 35 cycles of 30 s at 95ºC, 10 s at 56ºC, and 4 min at 60ºC. We purified tercycle products with multiscreen 96-well filtration plates (Millipore). The products were sequenced in 15 µl distilled water with an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

We designed three primer pairs (3 and 4; 5 and 6; and 7 and 8, see Table 1) based on the preliminary canine coding sequence. These pairs amplified overlapping products in PCR reactions containing 1 ng BAC 18L8 DNA. The first PCR product was produced in a 25 µl reaction volume with 1.5 mM $MgCl_{2,}$ 0.2 mM dNTPs, 0.5 µM primer 3 and 4, and 1.25 U Platinum® *Taq* DNA polymerase. The second PCR product was produced in a 50 µl reaction volume with 4 mM $MgCl_{2,}$ 0.34 mM dNTPs, 0.2 µM primer 5 and 6, and 0.5 U *Taq* DNA polymerase. The third PCR product was produced in a 20 µl reaction volume with 2.5 mM $MgCl_{2,}$ 0.2 mM dNTPs, 0.8 µM primer 7 and 8, and 0.7 U AmpliTaq Gold® DNA polymerase. We purified all PCR products with a QIAquick PCR purification kit. The DNA sequence of the PCR products was analysed with the automatic sequencer. We performed tercycle reactions in a 10 µl volume. The reactions contained 10 ng PCR product, 3.2 pmol primer, and 1 µl BigDye Terminator v3.0 Ready Reaction Mix in 1x sequence buffer (80 mM Tris, 2 mM $MgCl_2$, pH 9.0). Tercycle products were purified using multiscreen 96-well filtration plates (Millipore) and diluted several times before sequencing. We confirmed every base in the preliminary DNA sequence in this way. The 5´ and 3´ ends of the preliminary sequence were confirmed by repeated direct BAC DNA sequencing. Note that there is a mismatch in primer 5: the 10th base is a T, where the corresponding base in the exon is a C. This apparently did not affect the specificity of the primer.

We calculated homologies with human, murine and porcine coding sequences with MEGALIGN (LASERGENE Software, clustal W method). These DNA sequences have the following GenBank Accession Numbers: M89478 (human), M85151 (mouse) and AF188626 (pig). We derived predicted positions of the transmembrane regions and key amino acid residues in the protein from the SWISSPROT website (http://us.expasy.org/sprot/ ; Swissprot Accession Number P28222), the GPCRDB information system for G protein-coupled receptors (http://www.gpcr.org), and from several publications referred to in the relevant paragraphs.

## FISH mapping

Metaphase chromosomes were prepared from concanavalin A stimulated peripheral blood lymphocytes from karyotypically normal dogs. Fluorescence *in situ* hybridisation was performed on GTG-banded metaphase spreads as described by Zijlstra *et al.* (1997). Prior to FISH, well banded metaphases were captured using a Leica DMRA microscope equipped with the GENUS Image Analysis software of Applied Imaging. After capturing, slides were washed twice with 4x SSC/0.05% Tween20, dehydrated and air-dried. Total DNA of BAC clone 18L8 was used as probe and labelled with biotin-16-dUTP by nick-translation. Labelled BAC DNA was precipitated with a 50-fold excess of salmon ssDNA (Sigma) and a 100-fold excess of fragmented total dog DNA as competitor. The precipitate was resuspended in hybridisation solution containing 50% formamide/2x SSC/10% dextransulfate to a final probe concentration of 5 ng/µl. Specific sites of hybridisation were detected using avidin-FITC, and signals were amplified twice using additional layers of biotinylated goat anti-avidin and avidin-FITC. Chromosomes were counterstained with propidium iodide. Previously captured metaphases were re-examined after FISH, recaptured and analysed using the GENUS software. Chromosomes showing specific hybridisation signals were identified on the basis of their banding patterns in accordance with the recommendations of the Committee for the Standardised Karyotype of the Dog (*Canis familiaris)* (Switonski *et al.* 1996).

## Radiation hybrid mapping

We used the RHDF5000 canine whole genome radiation hybrid (RH) panel (Vignaux *et al.* 1999) for radiation hybrid mapping of the 18L8 clone. Primer pair 7 and 8 (Table 1) was used to establish retention of *htr1B* in the 126 hybrids. The primers were tested on genomic canine, hamster and 2:1

hamster/canine DNA in order to confirm their specificity for dog DNA. Conditions of the PCR reactions were as described above, except for the total reaction volume (10 µl instead of 20 µl). We used 50 ng hybrid DNA in each reaction.

We used the program "126to118" (http://www-recomgen.univ-rennes1.fr/Dogs/126to118.html) to convert the results to a 118 vector to be computed on the 3270-marker version of the RH map. The typing data were then incorporated into this map (Guyon *et al.* 2003), using the two-point analysis of the MULTIMAP package (Matise *et al.* 1994). RH maps for all chromosomes and additional information can be found at http://www-recomgen.univ-rennes1.fr/doggy.html.

**Microsatellite marker isolation and analysis**

In order to isolate a microsatellite marker from BAC 18L8, we subcloned 1 µg of BAC18L8 DNA as described above. Instead of hybridising zeocin-resistent colonies with probe *htr1B*-997, we randomly picked 96 colonies and grew them for six hours in 100 µl LB medium with 50 µg/ml zeocin. The cultures were diluted 10 times and we performed a PCR directly using M13 forward (5´-GTA AAA CGA CGG CCA GT - 3´) and reverse (5´- CAG GAA ACA GCT ATG AC - 3´) primers. We performed sequencing as described above with M13 forward primer. We blasted all DNA sequences on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) and examined them for the presence of microsatellite markers. A $(GA)_{13}$ repeat was identified.

In order to establish whether the marker is polymorphic, primers 9 and 10 (Table 1) were designed around the repeat. We tested the primers in the BAC, 2 Beagles, 2 Dobermanns and 6 Golden Retrievers. The 5´ end of primer 9 was labelled with 6-FAM fluorescent dye (Eurogentec). PCR reactions were performed in a 20 µl volume containing 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 µM of both primers, 0.7 U AmpliTaq Gold® DNA polymerase in 1x AmpliTaq Gold buffer. We used the following PCR program: 5 minutes initial denaturation at 95ºC, followed by 34 cycles of 30 seconds 94ºC, 30 seconds 57ºC, and 30 seconds 72ºC, followed by a final extension step of 2 minutes at 72ºC. We diluted labelled PCR products to a concentration of approximately 0.5 ng/µl. We mixed 2 µl of this dilution with 9.85 µl formamide and 0.15 µl TAMRA-GS500 size standard (Applied Biosystems) for analysis on the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The results from genomic DNA were compared to the results for BAC DNA in order to determine the accurate size of the fragments.

### Sequence analysis of *htr1B* in 2 Beagles, 2 Dobermanns and 6 Golden Retrievers

We analysed base pairs 7 - 1271 of *htr1B* in genomic DNA of two Beagles, two Dobermanns and six Golden Retrievers by DNA sequencing of PCR products, using primer pairs 3 and 4; 5 and 6; and 7 and 8. We used 25 ng of genomic DNA in each reaction. The PCR reactions and sequencing were performed as described above. We sequenced the 752 bp and 680 bp PCR products with alternative forward primers (5´- ACA TCC TCT ACA CCG TGT ACT C -3´ and 5´- GGT CAC CTC CGT TAA CTC G -3´ respectively) in some dogs because primer 5 and 7 did not give satisfying results.

## Results

### BAC library screening and Southern blot

BAC library screening with probe *htr1B*-997 resulted in six positive BAC clones: 1I5, 18L8, 86N14, 91D4, 163B17 and 164D24. All BAC DNA and genomic dog DNA showed a single *Eco*RI fragment of approximately 8 kb in a Southern blot analysis with the same probe. Southern blots of genomic dog DNA digested with *Bgl*II, *Bam*HI and *Pst*I also showed only one band (of respectively approximately 6.6 kb, 8 kb, and 2 kb). These results confirm the presence of *htr1B* on the BAC clones and imply that *htr1B* is likely to be a single-copy gene in the canine genome. We continued our experiment with clone 18L8.

### Sequence analysis of *htr1B*

We determined the entire coding sequence of *htr1B* on BAC clone 18L8, including 102 bp at the 5´-flanking side and 184 bp at the 3´-flanking side (Figure 1). Canine *htr1B* consists of one exon with an open reading frame (ORF) of 1170 bp. The nucleotide sequence in this ORF is highly similar to human, murine, and porcine *htr1B* sequences (91%, 89% and 91% identity, respectively). We discovered a 66 bp segment with 85% sequence homology to the human 5´ region in the canine 5´ flanking region. No homology was found with the murine 5´ sequence (canine nucleotides -68 up to -3, where the A in the ATG start codon is designated number 1). The 3´ flanking sequence contained a segment homologous to human and murine sequences (canine nucleotides 1179-1267 were 89% identical to the human sequence, and

CAAGCGGGACACCGGACTGTGGTATCTCCGCGGGCCTTCCGCCCTTTCCGTCGTTTGCTCCATGCCCCAGGGCTGCGCTC
CGGGGCCCCGGCGAGGAGAGAC

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ATG | GAA | GCA | GCC | GGC | GCT | CCG | TGC | GCC | CCG | CCG | CCG | CCC | GCG | GGC | TCC | CAG | ACC | 54 |
| M | E | A | A | G | A | P | C | A | P | P | P | P | A | G | S | Q | T | 18 |
| GGG | GCT | CCT | CCA | GCC | AAC | CTG | TCT | TCG | GCG | CCG | CAC | AAC | TGC | AGC | GCC | GAG | GGC | 108 |
| G | A | P | P | A | N | L | S | S | A | P | H | N | C | S | A | E | G | 36 |
| TAC | ATC | TAC | CAG | GAC | TCC | GTC | GCG | CTG | CCC | TGG | AAA | GTG | CTC | CTG | GTC | **A**TT | CTG | 162 |
| Y | I | Y | Q | D | S | V | A | L | P | W | K | V | L | L | V | I(L) | L | 54 |
| CTG | GCA | CTC | ATC | ACC | CTG | GCC | ACC | ACG | CTC | TCC | AAC | GCC | TTT | GTG | ATC | GCC | ACG | 216 |
| L | A | L | I | T | L | A | T | T | L | S | N | A | F | V | I | A | T | 72 |
| GTG | TAC | CGG | ACC | CGG | AAG | CTG | CAC | ACG | CC**G** | GCC | AAC | TAC | CTG | ATC | GCC | TCC | CTG | 270 |
| V | Y | R | T | R | K | L | H | T | P | A | N | Y | L | I | A | S | L | 90 |
| GCC | GTC | ACC | GAC | CTG | CTC | GTC | TCC | ATC | CTG | GTG | ATG | CCC | ATC | AGC | ACC | ATG | TAC | 324 |
| A | V | T | D | L | L | V | S | I | L | V | M | P | I | S | T | M | Y | 108 |
| ACG | GTC | ACC | GGC | CGC | TGG | ACG | CTG | GGC | CAG | GTG | GTC | TGC | GAC | TTG | TGG | CTG | TCG | 378 |
| T | V | T | G | R | W | T | L | G | Q | V | V | C | D | L | W | L | S | 126 |
| TCG | GAC | ATC | ACC | TGT | TGC | ACG | GCT | TCC | ATC | CTG | CAC | CTC | TGC | GTC | ATC | GCC | CTG | 432 |
| S | D | I | T | C | C | T | A | S | I | L | H | L | C | V | I | A | L | 144 |
| GAC | CGC | TAC | TGG | GCC | ATC | ACG | GAC | GCC | GTG | GAG | TAC | TCC | GCC | AAA | AGG | ACT | CCC | 486 |
| D | R | Y | W | A | I | T | D | A | V | E | Y | S | A | K | R | T | P | 162 |
| AAG | AGG | GCC | GCG | GTC | ATG | ATC | GCG | CTC | GTG | TGG | GTC | TTC | TCC | ATC | TCC | ATC | TCG | 540 |
| K | R | A | A | V | M | I | A | L | V | W | V | F | S | I | S | I | S | 180 |
| CTG | CCG | CCC | TTC | TTC | TGG | CGC | CAG | GCC | AAA | GCC | GAG | GAG | GAG | GTG | TCG | GAC | TGC | 594 |
| L | P | P | F | F | W | R | Q | A | K | A | E | E | E | V | S | D | C | 198 |
| GTG | GTG | AAC | ACC | GAC | CAC | ATC | CTC | TAC | ACC | GTG | TAC | TCC | ACG | GTG | GGC | GCT | TTC | 648 |
| V | V | N | T | D | H | I | L | Y | T | V | Y | S | T | V | G | A | F | 216 |
| TAC | TTC | CCC | AC**G** | CTG | CTC | CTC | ATC | GCC | CTC | TAC | GGC | CGC | ATC | TAC | GTG | GAA | GCC | 702 |
| Y | F | P | T | L | L | L | I | A | L | Y | G | R | I | Y | V | E | A | 234 |
| CGC | TCC | CGG | ATT | TTG | AAA | CAG | ACG | CCC | AAC | AGG | ACC | GGC | AAG | CGC | CTG | ACC | CGA | 756 |
| R | S | R | I | L | K | Q | T | P | N | R | T | G | K | R | L | T | R | 252 |
| GCC | CAG | CTG | ATA | ACC | GAC | TCC | CCC | GGC | TCC | ACG | TCC | TCG | GTC | ACC | TCC | GTT | AAC | 810 |
| A | Q | L | I | T | D | S | P | G | S | T | S | S | V | T | S | V | N | 270 |
| TCG | CGG | GCT | CCC | GAC | GTG | CCC | AGC | GAA | TCC | GGG | TCC | CCG | GTG | TAC | GTG | AAC | CAA | 864 |
| S | R | A | P | D | V | P | S | E | S | G | S | P | V | Y | V | N | Q | 288 |
| GTC | AAA | GTG | CGG | GTC | TCC | GAC | GCG | CTG | CTG | GAG | AAG | AAG | AAA | CTC | ATG | GCC | GCT | 918 |
| V | K | V | R | V | S | D | A | L | L | E | K | K | K | L | M | A | A | 306 |
| AGG | GAG | CGC | AAA | GCC | ACC | AAG | ACC | CTG | GGA | ATC | ATC | **C**TG | GGA | GCC | TTT | ATC | GTG | 972 |
| R | E | R | K | A | T | K | T | L | G | I | I | L | G | A | F | I | V | 324 |
| TGC | TGG | CTG | CCC | TTC | TTC | ATC | ATC | TCC | CTG | GTG | ATG | CCT | ATT | TGC | AAG | GAC | GCC | 1026 |
| C | W | L | P | F | F | I | I | S | L | V | M | P | I | C | K | D | A | 342 |
| TGC | TGG | TTC | CAC | CTG | GCC | ATC | TTC | GAC | TTC | TTC | ACG | TGG | CTG | GGC | TAT | CTC | AAC | 1080 |
| C | W | F | H | L | A | I | F | D | F | F | T | W | L | G | Y | L | N | 360 |
| TCC | CTT | ATC | AAC | CCC | ATC | ATC | TAT | ACC | ATG | TCC | AAT | GAG | GAC | TTC | AAA | CAA | GCG | 1134 |
| S | L | I | N | P | I | I | Y | T | M | S | N | E | D | F | K | Q | A | 378 |
| TTC | CAT | AAA | CT**C** | ATA | CGC | TTT | AAG | TGC | GCA | GGT | TGA | | | | | | | 1170 |
| F | H | K | L | I | R | F | K | C | A | G | | | | | | | | 389 |

CTTGTCAGTTGCAATGGGGTAGCCTGAGCGACCTTTGGGGACCATGCTGGGTCTGGTTCCACAGGTAGGTCGACTCTTCT
TTCACTGTTACTGGGTCAGGTTGAGGCTCTGTCTTCTGGCCAGTGGATCCTGAGAAGCCAGGACAGCCCTGAGGGCGCTC
CGCTCCCAGAGGAGACCTGTTCCA

**Figure 1.** Nucleotide sequence and deduced amino acid sequence of canine *htr1B*. The sequence has been deposited in the GenBank database under accession number AY323909. A preliminary sequence was obtained by means of sequencing subcloned BAC 18L8 with T7 and SP6 primers and direct BAC DNA sequencing. This sequence was confirmed by sequencing BAC DNA-based PCR products. Nucleotides printed in bold are polymorphic and the alternative amino acid is printed in brackets in case of an amino acid change.

1171-1266 were 86% identical to the murine sequence). The evolutionary conservation of these two segments suggests that they are involved in the function of the gene. The 5-$HT_{1B}$ protein consists of 389 amino acids in the dog, 390 amino acids in man and the pig, and 386 in the mouse. Canine amino acid identity is 95% with the human, 93% with the murine, and 94% with the porcine protein. The conservation in the transmembrane domains is high,

| | 1–10 | 11–20 | 21–30 | 31–40 | |
|---|---|---|---|---|---|
| DOG | MEAAGAPCAP | PPPAGSQTGA | PPANLSSAP* | HNCSAEGYIY | 39 |
| HUMAN | MEEPGAQCAP | PPPAGSETWV | PQANLSSAPS | QNCSAKDYIY | 40 |
| MOUSE | MEEQGIQCAP | PPPAASQTGV | PLTNLS**** | HNCSADGYIY | 36 |
| PIG | MEEAGAQCAP | PLPASSQTRL | SQANLSAAPS | QNCSAEGYIY | 40 |
| DOG | QDSVALPWKV | LLVILLALIT | LATTLSNAFV | IATVYRTRKL | 79 |
| HUMAN | QDSISLPWKV | LLVMLLALIT | LATTLSNAFV | IATVYRTRKL | 80 |
| MOUSE | QDSIALPWKV | LLVALLALIT | LATTLSNAFV | IATVYRTRKL | 76 |
| PIG | QDSIALPWKV | VLVILLALFT | LATTLSNAFV | IATVYRTRKL | 80 |
| DOG | HTPANYLIAS | LAVTDLLVSI | LVMPISTMYT | VTGRWTLGQV | 119 |
| HUMAN | HTPANYLIAS | LAVTDLLVSI | LVMPISTMYT | VTGRWTLGQV | 120 |
| MOUSE | HTPANYLIAS | LAVTDLLVSI | LVMPISTMYT | VTGRWTLGQV | 116 |
| PIG | HTPANYLIAS | LAVTDLLVSI | LVMPISTMYT | VTGRWTLGQV | 120 |
| DOG | VCDLWLSSDI | TCCTASILHL | CVIALDRYWA | ITDAVEYSAK | 159 |
| HUMAN | VCDFWLSSDI | TCCTASILHL | CVIALDRYWA | ITDAVEYSAK | 160 |
| MOUSE | VCDFWLSSDI | TCCTASIMHL | CVIALDRYWA | ITDAVEYSAK | 156 |
| PIG | VCDFWLSSDI | TCCTASILHL | CVIALDRYWA | ITDAVEYSAK | 160 |
| DOG | RTPKRAAVMI | ALVWVFSISI | SLPPFFWRQA | KAEEEVSDCV | 199 |
| HUMAN | RTPKRAAVMI | ALVWVFSISI | SLPPFFWRQA | KAEEEVSECV | 200 |
| MOUSE | RTPKRAAIMI | VLVWVFSISI | SLPPFFWRQA | KAEEEMLDCF | 196 |
| PIG | RTPKRAAVMI | ALVWVFSISI | SLPPFFWRQA | KAEEEVSDCV | 200 |
| DOG | VNTDHILYTV | YSTVGAFYFP | TLLLIALYGR | IYVEARSRIL | 239 |
| HUMAN | VNTDHILYTV | YSTVGAFYFP | TLLLIALYGR | IYVEARSRIL | 240 |
| MOUSE | VNTDHVLYTV | YSTVGAFYLP | TLLLIALYGR | IYVEARSRIL | 236 |
| PIG | VNTDHILYTV | YSTVGASYFP | TLLLIALYGR | IYVEARSRIL | 240 |
| DOG | KQTPNRTGKR | LTRAQLITDS | PGSTSSVTSV | NSRAPDVPSE | 279 |
| HUMAN | KQTPNRTGKR | LTRAQLITDS | PGSTSSVTSI | NSRVPDVPSE | 280 |
| MOUSE | KQTPNKTGKR | LTRAQLITDS | PGSTSSVTSI | NSRAPDVPSE | 276 |
| PIG | KQTPNRTGKR | LTRAQLITDS | PGSTSSVTSI | NSRAPDLPSE | 280 |
| DOG | SGSPVYVNQV | KVRVSDALLE | KKKLMAARER | KATKTLGIIL | 319 |
| HUMAN | SGSPVYVNQV | KVRVSDALLE | KKKLMAARER | KATKTLGIIL | 320 |
| MOUSE | SGSPVYVNQV | KVRVSDALLE | KKKLMAARER | KATKTLGIIL | 316 |
| PIG | SGSPVYVNQV | KVRVSDALLE | KKKLMAARER | KATKTLGIIL | 320 |
| DOG | GAFIVCWLPF | FIISLVMPIC | KDACWFHLAI | FDFFTWLGYL | 359 |
| HUMAN | GAFIVCWLPF | FIISLVMPIC | KDACWFHLAI | FDFFTWLGYL | 360 |
| MOUSE | GAFIVCWLPF | FIISLVMPIC | KDACWFHMAI | FDFFNWLGYL | 356 |
| PIG | GAFIVCWLPF | FIISLAMPIC | KDACWFHLAI | FDFFTWLGYL | 360 |
| DOG | NSLINPIIYT | MSNEDFKQAF | HKLIRFKCAG | | 389 |
| HUMAN | NSLINPIIYT | MSNEDFKQAF | HKLIRFKCTS | | 390 |
| MOUSE | NSLINPIIYT | MSNEDFKQAF | HKLIRFKCAG | | 386 |
| PIG | NSLINPIIYT | MFNEDFKQAF | HKLIRFKCTS | | 390 |

**Figure 2.** Alignment of the amino acid sequence of the canine serotonin receptor 1B with human (accession number M89478), murine (M85151), and porcine (AF188626) sequences. Amino acids are presented in groups of ten. Residues that are conserved in the four species are shown in grey background. Predicted transmembrane regions according to SWISSPROT are boxed. Residues 1-48 form the amino terminus; residues 73-85 form the first intracellular loop; etc. Key residues that are highlighted in the discussion are underlined: 121C and 198C (disulfide bridge); 387C (palmitoylation); 24N and 31N (glycosylation); 76T, 157S, 161T, 236S, 246T, 251T, 312T, and 371S (phosphorylation); and 354T (ligand binding). (All positions of key residues refer to the canine protein.) Amino acid residue 53 is depicted in a black box because it is polymorphic in the dog. This residue can be either isoleucine or leucine in dogs.

(Figure 2), although it does not compare with the homology in the serotonin receptor 1A gene, where these domains are 100% identical in dogs and humans (section 3.1).

## FISH mapping of BAC 18L8

For the chromosomal localisation of BAC clone 18L8, 27 GTG-banded metaphase spreads were analysed after fluorescence *in situ* hybridisation (FISH). Nineteen of these metaphases showed specific hybridisation signals on the chromosomes of pair twelve, band q1.6-1.7 (Figure 3). This is in agreement with previous localisation of *htr1B* in the dog using radiation hybrid mapping (Guyon *et al.* 2003).

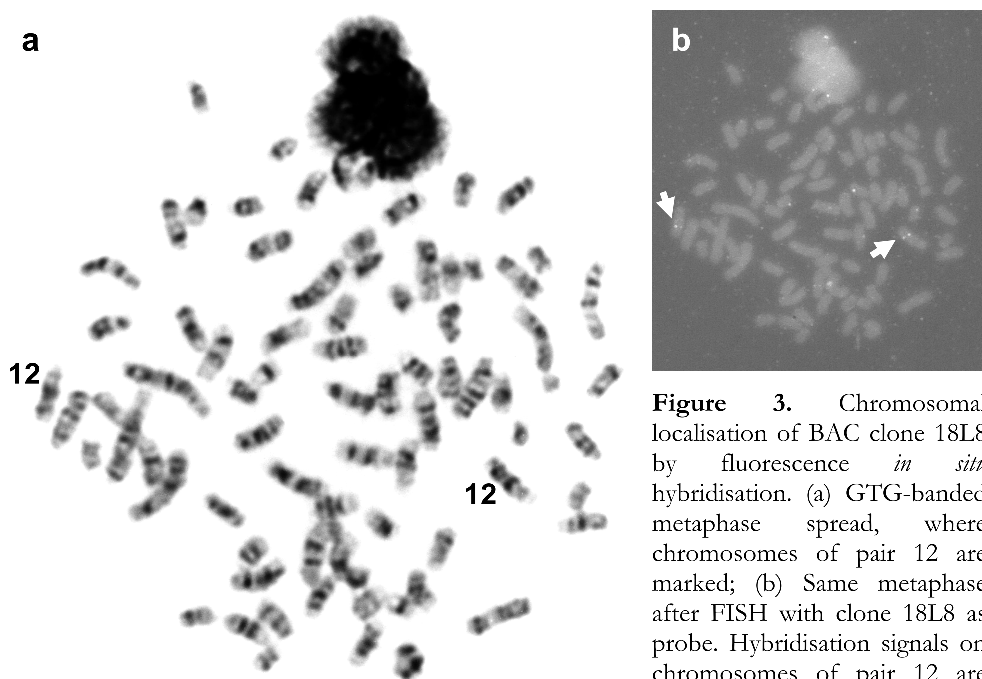


**Figure 3.** Chromosomal localisation of BAC clone 18L8 by fluorescence *in situ* hybridisation. (a) GTG-banded metaphase spread, where chromosomes of pair 12 are marked; (b) Same metaphase after FISH with clone 18L8 as probe. Hybridisation signals on chromosomes of pair 12 are marked with arrows.

## Radiation hybrid mapping

*Htr1B* was retained in 23% of the hybrids of the RHDF5000 panel. This is in accordance with its average retention rate of 21%. Computation of the vector on the 3270 RH map (Guyon *et al.* 2003) revealed that *htr1B* mapped on

CFA12 with high LOD scores with the previously mapped *htr1B* (LOD score = 22) and EST 5H8 (LOD score = 20).

### Microsatellite marker isolation and analysis

We sequenced 96 random subcloned fragments of BAC 18L8 in order to find microsatellite markers. The sequences were blasted and among the results was a 228 bp fragment with 84% identity to human clone RP11-551A13 on chromosome 6 (GenBank Accession Number AL390316). In addition, we identified a 22 bp fragment with 100% identity to the 3´UTR of human *htr1B* (GenBank Accession number D10995). Another sequence contained a $(GA)_{13}$ repeat. We have deposited the latter sequence in the GenBank database under accession number AY325269 and we have named this marker UU18L8. To determine whether the marker was polymorphic, it was analysed in 10 dogs (six Golden Retrievers, two Beagles, and two Dobermanns). We detected three alleles with product lengths of 139, 143, and 147 base pairs. The genotypes of the dogs are presented in Table 2.

### Sequence analysis of *htr1B* in Dobermanns, Beagles and Golden Retrievers

Sequence analysis of base pairs 7-1271 of *htr1B* in 10 different dogs revealed five single nucleotide polymorphisms (SNPs): A157C, G246A, C660G, T955C, and G1146C (Figure 1 and Table 2; note that the nucleotide with the highest evolutionary conservation is put in front in this notation, e.g. at the equivalent of position 157, human and porcine *htr1B* contain an A, while murine *htr1B* contains a G). All SNPs displayed variation between the six Golden Retrievers, indicating that they are not breed-specific. Table 2 reveals that there exist at least four haplotypes in the Golden Retrievers tested. Golden 3710 is homozygous for all markers, so the haplotype must be 143-C-A-C-T-G ("haplotype 1") in this dog. In the simplest situation Goldens 1264, 1265, 3031, and 3346 would also carry haplotype 1. Golden 1264 would then carry a second haplotype, 143-A-G-C-C-C ("haplotype 2"); Golden 1265 and 3031 would carry a third version, 143-A-G-C-T-G ("haplotype 3"); and Golden 3346 a fourth variant, 139-A-G-G-C-C ("haplotype 4"). Golden 3032 would carry haplotype 3 and 4.

Only A157C is expressed at the amino acid level. The other four SNPs are silent polymorphisms. A157C gives rise to an isoleucine/ leucine polymorphism of amino acid 53, which is situated in the amino terminus of the first transmembrane segment of the receptor. We do not expect this

polymorphism to have functional consequences because Ile and Leu have similar chemical properties.

**Table 2.** Genotypes of ten dogs for microsatellite marker UU18L8 (isolated from BAC 18L8) and five single nucleotide polymorphisms in the *htr1B* open reading frame.

| | UU18L8 | 157[b] | 246 | 660 | 955 | 1146 |
|---|---|---|---|---|---|---|
| **Golden 1264**[a] | 143/143 | A/C | A/G | C/C | C/T | G/C |
| **Golden 1265** | 143/143 | A/C | A/G | C/C | T/T | G/G |
| **Golden 3031** | 143/143 | A/C | A/G | C/C | T/T | G/G |
| **Golden 3032** | 139/143 | A/A | G/G | G/C | C/T | G/C |
| **Golden 3346** | 139/143 | A/C | A/G | G/C | C/T | G/C |
| **Golden 3710** | 143/143 | C/C | A/A | C/C | T/T | G/G |
| **Dobermann 5058** | 139/139 | A/A | G/G | C/C | T/T | G/G |
| **Dobermann 5126** | 139/139 | A/A | G/G | C/C | T/T | G/G |
| **Beagle 5260** | 139/147 | A/A | G/G | C/C | T/T | G/G |
| **Beagle 5265** | 139/143 | A/A | G/G | G/C | C/T | G/C |

[a] Numbers of the dogs refer to their numbers in our DNA database.
[b] Numbers of the single nucleotide polymorphisms refer to their position in *htr1B*.

# Discussion

## Canine *htr1B* sequence

We have isolated a canine BAC clone containing the gene encoding serotonin receptor 1B and we have determined the entire coding sequence of this gene. Sgard *et al.* (1996) previously published a partial sequence of *htr1B* (GenBank accession number S82461), running from position 573 to 1056 in the ORF. At that time, the gene was called *htr1Dß*. It is now generally accepted that *htr1Dß* is in fact a species variant of *htr1B* (Hartig *et al.* 1996). The Sgard *et al.* DNA sequence is completely identical to the DNA sequence published in this section, except at position 660 and 955 due to polymorphism of the gene.

Information about key functions of residues in the amino acid chain is indispensable in future studies on the association between mutations or polymorphisms in *htr1B* and behavioural disorders in dogs. The key ligand binding areas of G-protein coupled receptors are located mainly in the top one-third of transmembrane domains 3-7 (Hartig 1997). In particular, residue

354Thr in the serotonin receptor 1B is believed to be critical for ligand binding. The second and third intracellular loop appear to be important for coupling to the inhibition of adenylate cyclase (Albert and Tiberi 2001). In Figure 2, several specific key amino acid residues can be recognised: residues involved in a disulfide bridge (121Cys and 198Cys, SWISSPROT website http://us.expasy.org/sprot/ ; Swissprot Accession Number P28222), and potential sites for palmitoylation (387Cys), N-linked glycosylation (24Asp and 31Asp), phosphorylation by protein kinase C (76Thr, 157Ser, 161Thr, 236Ser and 246Thr), phosphorylation by tyrosine kinase (371Ser), and phosphorylation by protein kinase A (251Thr and 312Thr) (Jin *et al.* 1992; Hamblin *et al.* 1992; Maroteaux *et al.* 1992).

Most of the amino acid residues highlighted above are conserved in dogs, humans, mice and pigs, except for residues 354Thr and 371Ser. The presence of an asparagine residue at the equivalent of position 354 in the mouse is remarkable. Evolutionary conservation of key residues is usually high, because a mutation in such an amino acid is often deleterious and therefore removed from the population by natural selection (Majewski and Ott 2003). *Htr1B* illustrates an exception from the rule: the Thr354Asn mutation causes a marked difference in the pharmacology of murine and human 5-$HT_{1B}$ receptors, but it is not deleterious (Oksenberg *et al.* 1992).

## Localisation of canine *htr1B*

Radiation hybrid mapping of *htr1B* was previously performed with primers at positions 862-881 (forward) and 947-969 (reverse) in the *htr1B* ORF (Guyon *et al.* 2003). The PCR product we used was longer (680 bp) and our reverse primer was located in the 3´ non-coding flanking area. The high accordance between the two RH mapping results and the FISH mapping confirm the position of *htr1B*. In the human genome, *htr1B* maps to HSA 6q13. Homology between CFA12 and HSA6 has previously been demonstrated by reciprocal chromosome painting studies (Breen *et al.* 1999).

## Polymorphic markers

We identified six polymorphic markers, one microsatellite repeat and five SNPs, in this study. The four silent SNPs are located in conserved regions of the gene, whereas the SNP at nucleotide position 157 is located in a variable region of *htr1B*. The frequency of SNPs in the coding region of canine *htr1B* is remarkably high compared to the average of one in every 834 bp of coding region (Brouillette *et al.* 2000). A high frequency of polymorphisms has also

been reported for human *htr1B*: the coding sequence of the human gene contains nine SNPs, at nucleotide positions 129, 276, 371, 655, 705, 772, 861, 1099, and 1120 (Sanders *et al.* 2002). Future studies will have to reveal the frequency of the different alleles in various dog breeds.

The presence of at least four haplotypes in the small number of Golden Retrievers that we studied, suggests a large variation of the *htr1B* sequence within this breed. Dog breeds are considered to be genetically homogeneous (Ostrander *et al.* 2000). However, some dog breeds are more inbred than others, and the Golden Retriever breed has previously been reported to be relatively heterogeneous (Nielen *et al.* 2001). The haplotypes will be useful for genetic studies in dog populations for which behavioural data (for instance about aggressive behaviour) are available.

## Conclusion

In conclusion, we extended the available data on canine *htr1B* with its coding and flanking DNA sequence; developed primer sets for genomic sequencing of the coding region, and identified five single nucleotide polymorphisms within the ORF. In addition, the localisation of *htr1B* on the canine genomic map was confirmed by fluorescent *in situ* hybridisation (FISH) and radiation hybrid (RH) mapping, and we have isolated a nearby microsatellite marker. This work provides a starting point for mutation scans and association studies on dogs with behavioural problems.

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# 3.3

# 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*)

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## Abstract

We study canine serotonergic genes to investigate genetic factors underlying canine aggression. Here, we describe the characterization of three genes of the canine serotonergic system: the serotonin receptor 1A and 2A gene (*htr1A* and *htr2A*) and the serotonin transporter gene (*slc6A4*). We have isolated canine bacterial artificial chromosome clones containing these genes and we designed oligonucleotides for genomic sequencing of coding regions and intron-exon boundaries. Golden Retrievers were analysed for DNA sequence variations. We found two nonsynonymous single nucleotide polymorphisms (SNPs) in the coding sequence of *htr1A*, one SNP close to a splice site in *htr2A*, and two SNPs in *slc6A4*, one in the coding sequence and one close to a splice site. In addition, we identified a polymorphic microsatellite marker for each gene. *Htr1A* is a strong candidate for involvement in the domestication of the dog. We genotyped the *htr1A* SNPs in 41 dogs of seven breeds with diverse behavioural characteristics. At least three SNP haplotypes were found. Our results do not support involvement of the gene in domestication.

## Introduction

Aggression is the most frequently encountered behavioural problem in dogs, resulting in bite injuries reaching epidemic proportions (Beaver 1994; Lockwood 1995, Mikkelsen and Lund 2000; Wright and Nesselrote 1987). Aggression is influenced by both genetic and environmental factors. The nature, relative importance, and interaction of these factors are still poorly understood. To approach these questions, we have embarked on a study of the genetic factors underlying aggressive behaviour in dogs.

Abnormalities in human serotonin (5-HT) metabolism have been found in a variety of mental disorders, including pathological aggression and anxiety (Berman and Coccaro 1998; Lesch and Merschdorf 2000). There is evidence for a modulatory role of the serotonergic system in behavioural traits in dogs as well. For instance, Reisner *et al.* (1996) reported decreased concentrations of 5-hydroxyindoleacetic acid (the major metabolite of 5-HT) in cerebrospinal fluid of dominant aggressive dogs. Badino *et al.* (2004) found modifications of serotonergic receptor concentrations in the brains of aggressive dogs.

We aim to study the association of genes of the canine serotonergic system with aggression and fear in Golden Retriever dogs. The first step in these candidate gene studies is the analysis of the gene structure and variation. We have described the isolation and characterization of the canine serotonin receptor 1A and 1B genes (*htr1A* and *htr1B*) in section 3.1 and 3.2. Here, we describe the characterization of two additional genes of the canine serotonergic system: the genes encoding the serotonin receptor 2A (*htr2A*) and the serotonin transporter (solute carrier family member 6A4, *slc6A4*). Moreover, we have studied the canine *htr1A* in more detail.

The serotonin receptors 1A and 2A are G-protein coupled receptors with seven transmembrane domains. Several studies have suggested that the 1A receptor is associated with anxiety, depression, aggression, and stress response. For instance, *htr1A* knockout mice have shown increased anxiety in a number of experimental paradigms (Heisler *et al.* 1998; Parks *et al.* 1998; Ramboz *et al.* 1998). The association of polymorphisms in human *HTR2A* with neuropsychiatric disorders h!s been studied frequently. A mutation in human *HTR2A* (T102C) has been shown to be associated with altered 5-HT binding, which has been implicated in schizophrenia, suicidal behaviour, impaired impulse control, and aggression history (Abdolmaleky *et al.* 2004; Bjork *et al.* 2002; Khait *et al.* 2005). In addition, Peremans *et al.* (2003) found an increased binding index of serotonin 2A receptors in cortical brain regions of impulsive aggressive dogs.

The serotonin transporter, encoded by *slc6A4*, belongs to the family of sodium and chloride dependent transporters and it contains 12 transmembrane domains. The serotonin transporter is localised on the presynaptic membrane of serotonergic neurons and is responsible for the reuptake of 5-HT from brain synapses. The protein is a target for antidepressants and psycho stimulants (Barker and Blakely 1996; Feldman *et al.* 1997). A polymorphism in its promoter region influences serotonin transporter density in the brain and is associated with mental disorders in humans (Anguelova *et al.* 2003; Hariri *et al.* 2002; Katsuragi *et al.* 1999; Lesch *et al.* 1996; Lesch *et al.* 1999). *Slc6A4* knockout mice show reduced aggression and reduced home cage activity (Holmes *et al.* 2003).

The structure of these genes has been elucidated in humans and several other organisms. Human *HTR1A* is an intronless gene that maps to HSA5q11.2-q13 (Kobilka *et al.* 1987). *HTR2A* consists of three exons in humans and is located on HSA13q14-q21 (Chen *et al.* 1992; Hsieh *et al.* 1990; Saltzman *et al.* 1991; Sparkes *et al.* 1991; Stam *et al.* 1992). Human *SLC6A4* maps to HSA17q11.1-q12 and consists of 15 exons (Gelernter *et al.* 1995; Ramamoorthy *et al.* 1993). Exon 1 and 2 are non-coding (Lesch *et al.* 1994). Canine *htr1A* and *htr2A* have been cloned and sequenced previously (Masuda *et al.* 2004b; section 3.1 of this thesis).

We studied canine *htr1A* in more detail because the single nucleotide polymorphisms (SNPs) in this gene are nonsynonymous. *Htr1A* is a strong candidate for involvement in the process of domestication of wolves, because indications suggest that it plays a role in fearful behaviour. It could be hypothesised that specific variants of genes that modulate anxiety were selected for in the ancestors of the present-day domestic dogs. In that case, we would expect to find little variation in this region of the canine genome. We have therefore analysed the presence of *htr1A* SNP haplotypes in dogs from seven breeds with diverse behavioural characteristics.

## Materials and methods

### Animals and DNA isolation

All dogs included in this study were privately owned. The Golden Retrievers participated in our research project involving canine fear and aggression. The owners of these dogs considered them to be neither anxious, nor aggressive. Beagles, Boxers, Cairn Terriers, Dobermanns, Norwegian Elkhounds, and Shetland Sheepdogs were recruited from our clinical DNA bank. These dogs

were suffering from a somatic disease; their behavioural characteristics were not recorded. The breeds were selected because they have been reported to have diverse behavioural characteristics (Hart and Hart 1988). We selected dogs that had no known common grandparents. Dog and mouse (DBA/2) genomic DNA was isolated from whole blood leucocytes using the salt extraction method of Miller *et al.* (1988).

## BAC library screening

We designed two oligonucleotides (5´- TGC CAA TCC CAG TCT TCG GG -3´ and 5´- CAT GGA GCA GTC ATT AGC TGT CGG C -3´) based on exon 3 of murine *htr2A* (GenBank accession number: NM_172812). With these oligonucleotides, a 713-bp labelled probe (*htr2A*-713) was produced as described in section 3.2. For *slc6A4*, we used OVERGO MAKER (http://genome.wustl.edu/) to design pairs of overlapping oligonucleotides in exon 3 (5´- CTG AGC TTC ATC AAG GGG AAC GGG -3´ and 5´- TTC TTG CCC CAG GTC TCC CGT TCC -3´) and exon 13 (5´- AGG ATC TGC TGG GTG GCC ATC AGC -3´ and 5´- ACA GGA GAA ACA GAG GGC TGA TGG -3´) based on the human *SLC6A4* sequence NM_001045. The two 40-bp overgo probes (*slc6A4*-40.3 and *slc6A4*-40.13) were synthesised and labelled as described by Stabej *et al.* (2004). Screening of the canine genomic bacterial artificial chromosome (BAC) library RPCI-81 with these probes and BAC DNA isolation were performed as described previously (Li *et al.* 1999; section 3.2). To confirm the presence of the genes in the BAC clones, BAC DNA was subcloned and sequenced as described in section 3.2. (We have described the isolation of BAC clone 160O12 for canine *htr1A* in section 3.1.)

## *In silico* characterization of gene sequences

We performed a BLAST (basic local alignment search tool) search for *htr1A*, *htr2A*, and *slc6A4* sequences in dog-specific databases at the NCBI website (National Center for Biotechnology Information, http://www.ncbi.nih.gov/genome/seq/CfaBlast.html), using DNA sequences with accession numbers AY134445 (Dobermann *htr1A)*, NM_001005869 (Beagle *htr2A*), and NM_001045 (human *SLC6A4*) as queries. In addition, we blasted individual exons of the Boxer *slc6A4* sequence (ensembl gene ID ENSCAFG00000018990; http://www.ensembl.org/) against these databases. BLASTn was used to detect similarities between flanking regions of the genes in dogs and other mammals. We searched for CpG islands in the 5´ flanking regions with the WEBGENE CpG islands prediction tool (http://www.itba. mi.cnr.it/webgene/).

DNA sequences were imported into SeqMan (LASERGENE Software) and assembled. Regions with overlapping reads were inspected to find SNPs. Functional effects of polymorphisms were predicted with POLYPHEN (http://genetics.bwh.harvard.edu/cgi-bin/pph/polyphen.cgi). Furthermore, we searched for simple sequence repeats in flanking regions and mate pairs of traces (DNA sequence chromatograms) containing coding regions of the genes.

Similarities between *htr2A* or *slc6A4* and their orthologues in man, mouse, rat, pig, cow, and chicken were calculated with MegAlign (LASERGENE Software, clustal W method). These protein sequences have the following GenBank accession numbers: NP_000612 (human *HTR2A*), NP_766400 (murine *htr2A*), NP_058950 (rat *htr2A*), NP_999382 (porcine *htr2A*), NP_001036 (human *SLC6A4*), NP_034614 (murine *slc6A4*), CAA71909 (rat *slc6A4*), NP_777034 (bovine *slc6A4*), and NP_998737 (chicken *slc6A4*). Predicted positions of the transmembrane regions and key amino acid residues in the protein were derived from the SWISSPROT website (http://us.expasy.org/sprot/ ; Swissprot accession Number P28223 and P31645) and from several publications referred to in the relevant paragraphs. We have described the homologies between canine *htr1A* and its human and murine orthologue in section 3.1.

**DNA sequence analysis in Golden Retrievers**

We developed oligonucleotides to amplify the coding nucleotide sequences of the genes with polymerase chain reactions (PCRs, Table 1). The coding sequence of *htr1A,* with the exception of the first 14 nucleotides, was amplified by four overlapping PCRs with primers 1-8 (Table 1). Twenty-five µl PCR reactions contained 0.7 mM Tris-HCl, 0.67 mM $MgCl_2$, 1.0 mM mercapto-ethanol, 0.67 mM EDTA (pH=8.0), 1.66 mM $(NH_4)_2SO_4$, 0.5 U AmpliTaq DNA polymerase, 100 ng of both primers, 0.15 mg/ml BSA, 2.5 µl DMSO, 1.5 mM dNTPs, and 800 ng genomic DNA. The thermocycler profile was as follows: 4 min at 94ºC, followed by 35 cycles of 1 minute at 94ºC, 1 minute at $T_{A,}$ and 1 minute at 72ºC, concluded with a final extension step of 10 minutes at 72ºC. SNP genotyping in the dogs of seven breeds was performed by DNA sequencing of PCR products that were generated with primer pairs 1-2 and 5-6.

We used seven M13 tailed oligonucleotide pairs to amplify the three exons of canine *htr2A* (primers 9-22 in Table 1) and 16 M13 tailed oligonucleotide pairs to amplify the coding exons of canine *slc6A4* (primers 23-54 in Table 1). Parts of the flanking regions were also amplified. The PCR reactions contained 12.5 picomoles of each primer, 1.5 mM $MgCl_2$, 0.2 mM

**Table 1.** Primer pairs used in this study.

| Primer numbers | Sequence (5′ – 3′) [a] | Position [b] | Length of product (bp) [c] | $T_A$ (°C) [d] | DNA polymerase used [e] |
|---|---|---|---|---|---|
| 1 | GCA GGC ATG GAG GGG CTC AG | -6 to 14 | 447 | 60 | Ampli |
| 2 | CTT GTT CAC GTA GTC GAT GG | 422 to 441 | | | |
| 3 | TGT GCT GCA CCT CGT CCA TC | 353 to 372 | 535 | 60 | Ampli |
| 4 | TGC ACT TCA ATC ACC TCC AG | 868 to 887 | | | |
| 5 | ACA GTC AAG AAG GCG GAG AG | 685 to 704 | 391 | 60 | Ampli |
| 6 | GCC AGC AGA GGA TGA ACG TG | 1056 to 1075 | | | |
| 7 | GAT TGA AGT GCA CCG CGT GG | 876 to 895 | 430 | 60 | Ampli |
| 8 | ACC GGG CGG GCC TTC TCG TC | *14 to *33 | | | |
| 9 | CCA ACA AGA CTC CAC TAA CG | -219 to -200 | 680 | 57 | *Taq* |
| 10 | GCT GCT GCC ACT CAC CAT A | 409 to IVS1+15 | | | |
| 11 | CCT GAT GTC ACT TGC CAT AG | 336 to 355 | 311 | 57 | *Taq* |
| 12 | CAC TCT CCG GTG ATA ATA GG | IVS1+181 to +200 | | | |
| 13 | CCT CAC TGA CGC TAA CCT TC | IVS1-30 to -11 | 399 | 57 | *Taq* |
| 14 | TAC CTT GCG GCA ATG ACC AC | IVS2+115 to +134 | | | |
| 15 | ACC GTT GTG TAG CAG TGC TC | IVS2–71 to -52 | 425 | 57 | *Taq* |
| 16 | CAT AGT CCT CCT GCC GTA G | 915 to 933 | | | |
| 17 | CCA ACG ATC AAT CCA CAG G | 885 to 903 | 520 | 57 | *Taq* |
| 18 | GTA GGA GCG TCT TCT GAA TG | 1351 to 1370 | | | |
| 19 | AAC ACT ATA CCG GCC TTG G | 1231 to 1249 | 235 | 57 | *Taq* |
| 20 | GCT ATG GCA ACT GGT CTA TC | 1412 to *18 | | | |
| 21 | AGA AGA CGC TCC TAC AGA CA | 1356 to 1375 | 854 | 57 | *Taq* |
| 22 | AAG ACC ACG CTG GAG ATT G | *744 to *762 | | | |
| 23 | TGC GTA ACT CTG TTC TCC | -154 to -137 | 311 | 57 | *Taq* |
| 24 | GCC AGA CTC CAC CTT ATC | 106 to 123 | | | |
| 25 | GGA GCT ATC AGC ATG TAA GG | 30 to 49 | 428 | 60 | *Taq* |
| 26 | AGA CAT GAT CAC TGC TCT GG | IVS3+61 to +80 | | | |
| 27 | GTG AGG TCA TTC AAC ACA GG | IVS3-147 to -128 | 413 | 58 | Plat |
| 28 | CTG ATT CCA GAA GAA GGT CC | IVS4+78 to +97 | | | |
| 29 | TTA CCA CAT TGC CAC CTG | IVS4-146 to -129 | 461 | 62 | Plat |
| 30 | TTC CTC GGA AGC CAA GTC | IVS5+44 to +61 | | | |
| 31 | AGG AGT TCC TAA GGC TGG TC | IVS5-130 to -111 | 391 | 57 | *Taq* |
| 32 | TCT GTG GCT GTC CAG GAT AC | IVS6+69 to +88 | | | |
| 33 | CCT GCC TCC TAT AGT TAC | IVS6-123 to -106 | 329 | 57 | *Taq* |
| 34 | GAC AGA CAG GTG CAC ATC | IVS7+20 to +37 | | | |
| 35 | TTG CAC TTG GTA TGT GGC TG | IVS7-127 to -108 | 456 | 62 | Plat |
| 36 | TCA ATC TCT GAA TGG CCT GG | IVS8+172 to +191 | | | |
| 37 | CAG TTC ACA ACA GGA CCA TC | IVS8-242 to -223 | 451 | 57 | *Taq* |
| 38 | AGC AAC TCA GTG AGA GCA AG | IVS9+28 to +47 | | | |
| 39 | TCA TTG TTG GTG TGG CTG AG | IVS9-90 to -71 | 408 | 57 | *Taq* |
| 40 | TCA AGA GCA CCA CAG TGA GG | IVS10+152 to +171 | | | |
| 41 | CTA CTC ATG ACC AGC AAC | IVS10-98 to -81 | 533 | 57 | *Taq* |
| 42 | CCA GAT ACT CTG TCA AGC | IVS11+252 to +269 | | | |
| 43 | AGT GCT CCA TAG GAC AGG | IVS11-202 to -185 | 529 | 60 | Plat |
| 44 | TTG TGG TAG AGC GTG AAG | IVS12+176 to +193 | | | |
| 45 | CGT CTC AAC TTC AGA GCA G | IVS12-141 to -123 | 587 | 57 | *Taq* |
| 46 | GAT GTG ACA CAT GCA GCA G | IVS13+293 to +311 | | | |

**Table 1, continued.**

| Primer numbers | Sequence (5´ – 3´) [a] | Position [b] | Length of product (bp) [c] | $T_A$ (°C) [d] | DNA polymerase used [e] |
|---|---|---|---|---|---|
| 47<br>48 | TCA GAA CTG TCT GCC AGG<br>CCA CTG CAT CTA AGG CTC | IVS13-61 to -44<br>IVS14+176 to +193 | 456 | 56 | Plat |
| 49<br>50 | GTC ACA TTG TCC AAC TGA GC<br>GTC ATT GGA GGC CAT AAG AG | IVS14-224 to -205<br>*163 to *182 | 515 | 57 | *Taq* |
| 51<br>52 | AGT CAT GCC TCA CCT TCA CC<br>TCC TGA CTC CAC AGC AGC AC | *56 to *75<br>*514 to *533 | 512 | 60 | Plat |
| 53<br>54 | ATG TGT GAG GCT GTG TAT GG<br>GGC AGA GCA TGT TGT AGT AG | *254 to *273<br>*704 to *723 | 504 | 60 | Plat |
| 55<br>56 | CCT CTA TCT CAG CAC TTG<br>GCT AAC ACC AGA GGA ACC | downstream of<br>*htr1A* | ± 297 | 53 | Gold |
| 57<br>58 | ACT GTT GAC TGA CCG CCT AC<br>GCT TCA TTC TCT CGC TCC TAC | IVS2+1397 to+1416<br>IVS2+1510 to+1530 | ±134 | 57 | *Taq* |
| 59<br>60 | TGT GGT GAC CGA TGA CAG<br>CAG GTG GCA ATG TGG TAA | IVS4-392 to -375<br>IVS4-146 to -129 | ±264 | 52 | *Taq* |

[a] Primers 9-54 had M13 sequence primer tails (not shown in the table).
[b] We used the nomenclature recommended by den Dunnen and Antonarakis (2001): the A of the ATG start codon is designated number 1; the nucleotide 5´ to this A is numbered –1; and the nucleotide 3´ of the translation termination codon is *1. Positions in introns refer to the nearest exon. The nomenclature of the introns is based on the human gene structure. IVS= intervening sequence.
[c] Product length includes M13 tail for primers 9-54.
[d] $T_A$ = annealing temperature
[e] Ampli = AmpliTaq DNA polymerase (Applied Biosystems); *Taq* = *Taq* DNA polymerase (Invitrogen); Plat = Platinum *Taq* DNA polymerase (Invitrogen); Gold = AmpliTaq Gold DNA polymerase (Applied Biosystems).

dNTPs, 1.25 U DNA polymerase, and 25 ng genomic DNA in a 25 µl reaction with Gibco-BRL buffer. The thermocycler profile was as follows: 3 min at 94ºC, followed by 35 cycles of 30 s at 94ºC, 30 s at $T_{A,}$ and 30 s at 72ºC, concluded with a final extension step of 4 minutes at 72ºC.

PCR products were purified with a QIAquick PCR purification kit and the DNA sequence was analysed with an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) as described in section 3.2. *Htr1A* PCR products were sequenced with 3.2 picomoles of one of the primers 1-8. All *htr2A* and *slc6A4* PCR products contained M13 tails and were sequenced with 3.2 picomoles of HPLC purified M13 forward (5´- GTT TTC CCA GTC ACG

AC -3´), or reverse (5´- CAG GAA ACA GCT ATG AC -3´) primer (Eurogentec). The chromatograms were inspected by eye in order to detect heterozygotes.

### Microsatellite marker genotyping

We analysed one polymorphic CA dinucleotide repeat for each gene with oligonucleotides 55-56 (*htr1A*), 57-58 (*htr2A*), and 59-60 (*slc6A4*). The 5´ end of the forward primer was labelled with 6-FAM fluorescent dye (Eurogentec). PCRs and automated analysis of the PCR products were performed as described in section 3.2. We used either 500-LIZ or TAMRA-GS500 as size standard (Applied Biosystems). GENESCAN 3.7 software was used for genotype assessment. The oligonucleotides for the *htr1A* microsatellite marker were also tested on BAC160O12 DNA since this canine BAC clone contains *htr1A*. This PCR product was sequenced in order to confirm the identity of the fragment.

## Results

### Isolation of canine BAC clones containing *htr2A* or *slc6A4*

BAC library screening with probe *htr2A*-713 resulted in two positive BAC clones: 351G1 and 422M22. A 0.63 kb DNA sequence read from one of the 351G1 subclones displayed 98% homology to position 890 to *106 of the canine gene (*106 means 106 bp 3´ of the stop codon). Comparison of this DNA sequence with the dog genome assembly resulted in only one hit: a chromosome 22 genomic contig (accession number NW_139892). Further analysis showed that this contig contains the complete canine *htr2A*. This result confirms the presence of exon 3 of *htr2A* in BAC clone 351G1 and implies only one copy of the 0.63 kb fragment in the canine genome.

BAC library screening with the overgo probes *slc6A4*-40.3 and *slc6A4*-40.13 resulted in thirteen positive BAC clones: 16P8, 45A9, 59N4, 76G6, 85K12, 202E23, 273H2, 281C3, 287M9, 307D21, 342D19, 356B20, and 326I14. The inserts of clones 76G6 and 287M9 were subcloned and randomly sequenced. One of the 76G6 subclones contained a 0.8 kb DNA sequence containing a CA repeat. This repeat is located at position IVS4-216 in intron 4 of canine *slc6A4* and we named the marker UU76G6. Comparison of the 0.8 kb DNA sequence containing the marker against the dog genome database retrieved a single hit with high similarity on CFA09 (accession number

NW_139866). Further analysis showed that this contig contains the complete canine *slc6A4*, as shown in the paragraph concerning the *in silico* characterization of *slc6A4*. UU76G6 was demonstrated by PCR to be present also in clone 287M9 (data not shown). These results confirm the presence of intron 4 of *slc6A4* in the two BAC clones.

### ***In silico*** **characterization of** ***htr1A***

The BLAST search with Dobermann *htr1A* sequence AY134445 retrieved one chromosome 2 genomic contig and 15 traces from the Boxer genome project, two sequences from the Poodle genome project, and a sequence from a Collie dog (Kukekova *et al.* 2004). The relative position and accession numbers of these sequences are depicted in Figure 1A. We found several segments with high homology to corresponding human and murine regions in the sequence upstream of the *htr1A* start codon. For instance, there is a 113 bp segment with 85% identity to the murine 5´ region of *htr1A* located at 3.5 kb upstream of the start codon. This sequence is also highly similar to the corresponding human region. In addition, a 298 bp segment with 80% identity to the human 5´ region of *HTR1A* was located 0.4 to 0.1 kb upstream of the start codon.

Two SNPs were identified in the coding sequence of *htr1A* by comparing the canine sequences: G65T and A808C. The latter was already reported in section 3.1. Both SNPs are nonsynonymous. G65T causes an arginine/leucine polymorphism of amino acid 22. A808C gives rise to a lysine/glutamine polymorphism of amino acid 270. The variations were predicted to be functionally insignificant by POLYPHEN.

A CA microsatellite repeat was found in a trace located downstream of *htr1A* (Figure 1A). A PCR indicated that this repeat was present in BAC clone 160O12, which has been shown to contain *htr1A* (data not shown; see section 3.1). The presence of the CA repeat in this clone confirms its proximity to the gene. We have named the marker UU160O12.

### ***In silico*** **characterization of** ***htr2A***

Using the Beagle *htr2A* sequence NM_001005869 as a BLAST query, we retrieved the earlier mentioned chromosome 22 genomic contig NW_139892 and 32 traces from the Boxer genome project, four sequences from the Poodle genome project, and a partial mRNA sequence of canine *htr2A* (Figure 1B). We detected a fragment of 38 bp with at least 90% identity to corresponding regions in human, hamster, cow, mouse, and rat at 0.9 kb upstream of the start codon. One kb 5´ sequence and the first 307 bp of the coding sequence are

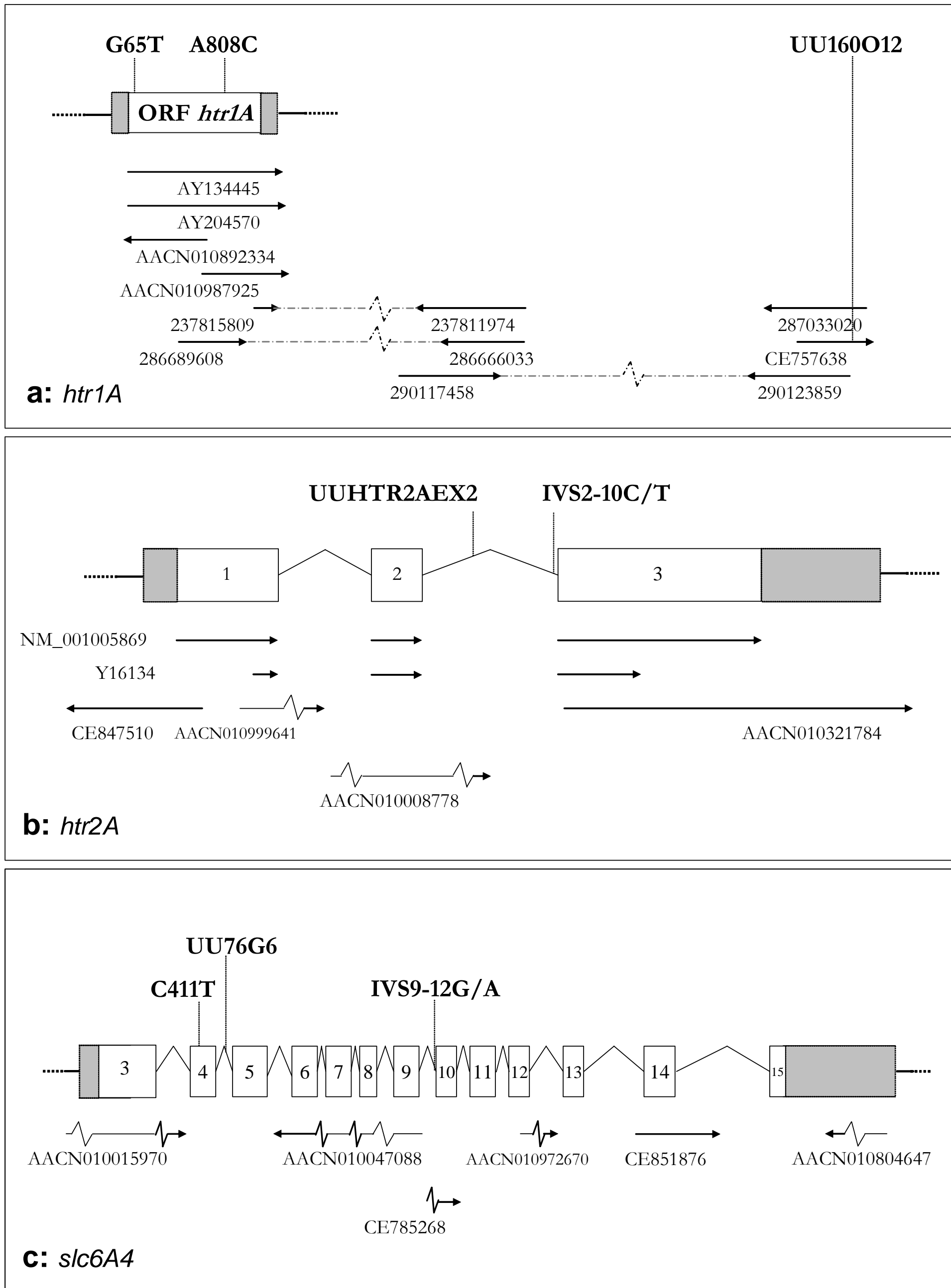


**Figure 1.** Position of retrieved canine DNA sequences relative to each other and to the exons of three serotonergic genes. The directions of the arrows represent the orientation of the sequences. Boxes mark the position of the exons. White areas represent coding sequence; grey areas represent flanking untranslated regions. The size of the untranslated regions and the numbers of the exons are based on the structure of the human genes, which does not necessarily correspond to the structure of the canine orthologues of the

◄ genes. Dotted vertical lines indicate the position of the polymorphic microsatellite markers and single nucleotide polymorphisms that were analysed in Golden Retrievers in this study. Introns and flanking regions are not drawn to scale.
**a.** *Htr1A* sequences: AY134445 is a Dobermann *htr1A* sequence, AY204570 is derived from a Collie, AACN010892334 and AACN010987925 are derived from a Poodle, and the sequences with numbers are traces retrieved from the Boxer genome project. Trace 237815809 and 237811974 form a mate pair containing sequences from the ends of a genomic DNA clone. The same applies to trace 286689608 - 286666033 and 290117458 - 290123859. Trace 290123859 and 287033020 contain a CA repeat, which is also present in the Poodle sequence CE757638. Thirteen additional traces that overlap with the *htr1*A open reading frame (ORF) are omitted in this figure. The 45.4 Mb Boxer chromosome 2 genomic contig NW_139841 spans the entire region and is not shown here.
**b.** *Htr2A* sequences: NM_001005869 is a cDNA sequence from a Beagle; Y16134 is a partial cDNA sequence of a dog of unspecified breed. CE847510, AACN010999641, AACN010008778, and AACN010321784 are Poodle DNA sequences. Thirty-two Boxer traces that overlap with the *htr2A* coding sequence are not shown. The 55.6 Mb Boxer chromosome 22 contig NW_139892 spans the entire region (not shown). A CpG enriched island is located about the start codon at position –1019 to 307 (not shown).
**c.** *Slc6A4* sequences: AACN010015970, AACN010047088, CE785268, AACN010972670, CE851876, and AACN010804647 are Poodle DNA sequences. More than 100 Boxer traces that overlap with the *slc6A4* coding sequence are not depicted. The 37.3 Mb Boxer contig NW_139866 spans the entire region (not shown). The non-coding exons 1 and 2 are not shown in this figure.

part of a CpG island according to the definition of Milanesi and Rogozin (1998).

We searched for polymorphisms within or close to *htr2A* in the retrieved DNA sequences. No variation was observed in the coding sequence. We found a G/A SNP at position IVS1+405, a T/C SNP at position IVS2+444, and a G/A SNP at IVS2+450. Several polymorphic simple sequence repeats were identified, including two CA repeats and an ATTT repeat in intron 2 and two A repeats downstream of the stop codon. One of the CA repeats was analysed in Golden Retrievers in this study (discussed later). This marker is located at position IVS2+1439 and we have named it UUHTR2AEX2.

The serotonin receptor 2A protein consists of 470 amino acids in dog and pig and of 471 amino acids in human, mouse, and rat. An alignment of these amino acid sequences is shown in Figure 2. Canine amino acid sequence identity is 94% with human, 90% with mouse and rat and 94% with pig. *HTR2A* protein sequence similarity is especially high in the seven transmembrane domains. This has also been observed in other G-protein coupled receptors (Stam *et al.* 1992). Identification of key residues in the amino acid chain is important for future studies on the association of mutations or polymorphisms in the genes with behavioural traits in dogs. The key ligand

| Species | | | | | Position |
|---|---|---|---|---|---|
| DOG | MDVLFEDNAP | LSPTTSSLMP | SNGDPRLYGN | DLNAGDANTS | 40 |
| HUMAN | MDILCEENTS | LSSTTNSLMQ | LNDDTRLYSN | DFNSGEANTS | 40 |
| PIG | MDVLCEENTS | LSSPTNSFMQ | LNDDTRLYHN | DFNSGEANTS | 40 |
| MOUSE | MEILCEDNIS | LSSIPNSLMQ | LGDDSRLYPN | DFNSRDANTS | 40 |
| RAT | MEILCEDNIS | LSSIPNSLMQ | LGDGPRLYHN | DFNSRDANTS | 40 |
| DOG | DAFNWTVDAE | NRTNLSCEGC | LSPPCFSLLH | LQEKNWSALL | 80 |
| HUMAN | DAFNWTVDSE | NRTNLSCEGC | LSPSCLSLLH | LQEKNWSALL | 80 |
| PIG | DAFNWTVDSE | NRTNLSCEGC | LSPPCFSLLH | LQEKNWSALL | 80 |
| MOUSE | EASNWTIDAE | NRTNLSCEGY | LPPTCLSILH | LQEKNWSALL | 80 |
| RAT | EASNWTIDAE | NRTNLSCEGY | LPPTCLSILH | LQEKNWSALL | 80 |
| DOG | TAVVIILTIA | GNILVIMAVS | LEKKLQNATN | YFLMSLAIAD | 120 |
| HUMAN | TAVVIILTIA | GNILVIMAVS | LEKKLQNATN | YFLMSLAIAD | 120 |
| PIG | TAVVIILTIA | GNILVIMAVS | LEKKLQNATN | YFLMSLAIAD | 120 |
| MOUSE | TTVVIILTIA | GNILVIMAVS | LEKKLQNATN | YFLMSLAIAD | 120 |
| RAT | TTVVIILTIA | GNILVIMAVS | LEKKLQNATN | YFLMSLAIAD | 120 |
| DOG | MLLGFLVMPV | SMLTILYGYR | WPLPSKLCAV | WIYLDVLFST | 160 |
| HUMAN | MLLGFLVMPV | SMLTILYGYR | WPLPSKLCAV | WIYLDVLFST | 160 |
| PIG | MLLGFLVMPV | SMLTILYGYR | WPLPSKLCAV | WIYLDVLFST | 160 |
| MOUSE | MLLGFLVMPV | SMLTILYGYR | WPLPSKLCAV | WIYLDVLFST | 160 |
| RAT | MLLGFLVMPV | SMLTILYGYR | WPLPSKLCAI | WIYLDVLFST | 160 |
| DOG | ASIMHLCAIS | LDRYVAIQNP | IHHSRFNSRT | KAFLKIIAVW | 200 |
| HUMAN | ASIMHLCAIS | LDRYVAIQNP | IHHSRFNSRT | KAFLKIIAVW | 200 |
| PIG | ASIMHLCAIS | LDRYVAIQNP | IHHRRFNSRT | KAFLKIIAVW | 200 |
| MOUSE | ASIMHLCAIS | LDRYVAIQNP | IHHSRFNSRT | KAFLKIIAVW | 200 |
| RAT | ASIMHLCAIS | LDRYVAIQNP | IHHSRFNSRT | KAFLKIIAVW | 200 |
| DOG | TISVGISMPI | PVFGLQDDSK | VFKEGSCLLA | DDNFVLIGSF | 240 |
| HUMAN | TISVGISMPI | PVFGLQDDSK | VFKEGSCLLA | DDNFVLIGSF | 240 |
| PIG | TISVGISMPI | PVFGLQDDSK | VFKEGSCLLA | DDNFVLIGSF | 240 |
| MOUSE | TISVGISMPI | PVFGLQDDSK | VFKEGSCLLA | DDNFVLIGSF | 240 |
| RAT | TISVGISMPI | PVFGLQDDSK | VFKEGSCLLA | DDNFVLIGSF | 240 |
| DOG | VSFFIPLTIM | VITYFLTIKS | LQKEATLCVS | DPGTRAKLAS | 280 |
| HUMAN | VSFFIPLTIM | VITYFLTIKS | LQKEATLCVS | DLGTRAKLAS | 280 |
| PIG | VSFFIPLTIM | VITYFLTIKS | LQKEATLCVS | DLGTRAKLAS | 280 |
| MOUSE | VAFFIPLTIM | VITYFLTIKS | LQKEATLCVS | DLSTRAKLSS | 280 |
| RAT | VAFFIPLTIM | VITYFLTIKS | LQKEATLCVS | DLSTRAKLAS | 280 |

| Species | | | | | Position |
|---|---|---|---|---|---|
| DOG | F S F L P Q S S L S | S E K L F Q R S I H | R E P G S Y - G R R | T M Q S I S N E Q K | 319 |
| HUMAN | F S F L P Q S S L S | S E K L F Q R S I H | R E P G S Y T G R R | T M Q S I S N E Q K | 320 |
| PIG | F S F L P Q S S L S | S E K L F Q R S I H | R E P G S Y - G R R | T M Q S I S N E Q K | 319 |
| MOUSE | F S F L P Q S S L S | S E K L F Q R S I H | R E P G S Y A G R R | T M Q S I S N E Q K | 320 |
| RAT | F S F L P Q S S L S | S E K L F Q R S I H | R E P G S Y A G R R | T M Q S I S N E Q K | 320 |
| DOG | A C K V L G I V F F | L F V V M W C P F F | I T N I M A V I C K | E S C N E D I I G A | 359 |
| HUMAN | A C K V L G I V F F | L F V V M W C P F F | I T N I M A V I C K | E S C N E D V I G A | 360 |
| PIG | A C K V L G I V F F | L F V V M W C P F F | I T N I M A V I C K | E S C N E D V I G A | 359 |
| MOUSE | A C K V L G I V F F | L F V V M W C P F F | I T N I M A V I C K | E S C N E N V I G A | 360 |
| RAT | A C K V L G I V F F | L F V V M W C P F F | I T N I M A V I C K | E S C N E N V I G A | 360 |
| DOG | L L N V F V W I G Y | L S S A V N P L V Y | T L F N K T Y R S A | F S R Y I Q C Q Y K | 399 |
| HUMAN | L L N V F V W I G Y | L S S A V N P L V Y | T L F N K T Y R S A | F S R Y I Q C Q Y K | 400 |
| PIG | L L N V F V W I G Y | L S S A V N P L V Y | T L F N K T Y R S A | F S R Y I Q C Q Y K | 399 |
| MOUSE | L L N V F V W I G Y | L S S A V N P L V Y | T L F N K T Y R S A | F S R Y I Q C Q Y K | 400 |
| RAT | L L N V F V W I G Y | L S S A V N P L V Y | T L F N K T Y R S A | F S R Y I Q C Q Y K | 400 |
| DOG | E N K K P L Q L I L | V N T I P A L A Y K | S S Q L Q M G Q K K | N S K K D A K S T D | 439 |
| HUMAN | E N K K P L Q L I L | V N T I P A L A Y K | S S Q L Q M G Q K K | N S K Q D A K T T D | 440 |
| PIG | E N K K P L Q L I L | V N T I P A L A Y K | S S Q L Q T G Q K E | N S K Q D D K A T E | 439 |
| MOUSE | E N R K P L Q L I L | V N T I P T L A Y K | S S Q L Q V G Q K K | N S Q E D A E P T A | 440 |
| RAT | E N R K P L Q L I L | V N T I P A L A Y K | S S Q L Q V G Q K K | N S Q E D A E Q T V | 440 |
| DOG | N D Y S M V A L G K | Q H S E D A P T D N | I N T V N E K V S C | V | 470 |
| HUMAN | N D C S M V A L G K | Q H S E E A S K D N | S D G V N E K V S C | V | 471 |
| PIG | N D C T M V A L G K | Q H S E D A P A D N | S N T V N E K V S C | V | 470 |
| MOUSE | N D C S M V T L G N | Q H S E E M C T D N | I E T V N E K V S C | V | 471 |
| RAT | D D C S M V T L G K | Q Q S E E N C T D N | I E T V N E K V S C | V | 471 |

**Figure 2.** Alignment of the amino acid sequence of the canine serotonin receptor 2A with human (accession number NP_000612), porcine (NP_999382), murine (NP_766400), and rat (NP_058950) sequences. Amino acids are presented in groups of ten. Residues that are conserved in the five species are shown in grey background and predicted transmembrane (TM) regions according to SWISSPROT (http://us.expasy.org/cgi-bin/niceprot.pl?P28223) are boxed. The seven TM domains are predicted to form a pocket where 5-HT can bind. Residues 1-75 form the amino terminus; residues 100-110 form the first intracellular loop, and so on. Key residues that are highlighted in the results paragraph are underlined: 8N, 38N, 44N, 51N, 54N are N-linked glycosylation sites, 148C and 227C form a disulfide bridge according to SWISSPROT. All positions of key residues refer to the canine protein.

binding areas of G-protein coupled receptors are located mainly in the distal one-third of transmembrane domains 3-7 (Hartig 1997). In Figure 2, several specific key amino acid residues in the serotonin receptor 2A protein can be recognised: residues involved in N-linked glycosylation (8Asn, 38Asn, 44Asn, 51Asn, and 54Asn) and in a disulfide bridge (148Cys and 227Cys) (SWISSPROT website http://expasy.org/sprot/ ; accession number P28223). These residues are conserved between the five species.

## *In silico* characterization of *slc6A4*

We retrieved the earlier mentioned chromosome 9 genomic contig, more than 100 traces, and six Poodle sequences by blasting the human *SLC6A4* sequence NM_001045 (Figure 1C). A region with 78% identity to the noncoding exon 1 of human *SLC6A4* was found at 12.5 kb upstream of the start codon. This region is part of a CpG island according to the definition of Milanesi and Rogozin (1998). The region is therefore likely to represent the first exon of the canine gene. We detected a region with 52% identity to the non-coding exon 2 of human *SLC6A4* at 0.1 kb upstream of the start codon. This region is flanked by a putative splice acceptor AG and donor GT site. We have adopted the human nomenclature of the exons in this article: the canine exon containing the ATG start codon is referred to as exon 3.

We found a single SNP at position –2200 by comparison of the Poodle and Boxer sequences, but we could not confirm this variation with traces. The serotonin transporter protein consists of 630 amino acids in dog, human, mouse, rat, and cow and of 670 amino acids in chicken (Figure 3). The canine amino acid homology is 94% with human, 91% with mouse and cow, 90% with rat, and 79% with chicken. In Figure 3, several specific key amino acid residues of *slc6A4* can be recognised: residues involved in N-linked glycosylation (208Asn and 217Asn; SWISSPROT website http:// expasy.org/sprot/ ; accession number P31645), in a disulfide bridge (200Cys and 209Cys; Chen *et al.* 1997), in binding of 5-HT (172Ile and 176Tyr; Chen and Rudnick 2000), and in the interaction with antagonists (95Tyr and 586Phe; Barker and Blakely 1996; Barker *et al.* 1998). Amino acids 8Ser, 13Ser, 277Ser, and 603Thr were marked as potential sites of protein kinase A and C phosphorylation by Ramamoorthy *et al.* (1993) and Chang *et al.* (1996). Chen and Rudnick (2000) suggested that 179Ile acts as part of an external gate. Most of the amino acid residues described here are conserved between the six species, except for 172Ile and 586Phe.

## Genotyping of Golden Retrievers

DNA sequence analysis of position 15 to *13 of *htr1A* was performed in three Golden Retrievers. This fragment did not display variation in the Retrievers. The dogs were homozygous for a T-residue at position 65 and homozygous for a C at position 808. We found three alleles of the marker UU160O12 in these dogs, with a PCR product length of 297, 303, and 305 bp. Each of the three dogs was heterozygous.

*Htr2A* DNA sequence analysis and marker genotyping were performed in eight Golden Retrievers. There was no variation in the coding sequence of *htr2A* in these dogs. However, we did find a C/T SNP at position IVS2-10. This polymorphism was not detected in the canine sequences that we retrieved from the NCBI website. Five dogs were heterozygous and three dogs were homozygous for the C allele. We found two alleles for the marker UUHTR2AEX2 with a length of 130 and 132 bp. Three combinations of the SNP and microsatellite marker alleles were observed in the Golden Retrievers (130-C, 132-C, and 132-T).

DNA sequence analysis of *slc6A4* was performed in eight Golden Retrievers. We identified a synonymous SNP in the coding sequence: C411T. In addition, we found a G/A SNP at position IVS9-12. Both SNPs were not detected in the canine sequences that we retrieved from the NCBI website. Four dogs were homozygous at both loci; these dogs had two copies of haplotype C-G of the two SNPs combined. The other four dogs were heterozygous at both positions; their haplotypes are likely to be C-G and T-A. We tested the marker UU76G6 in seven Golden Retrievers and found two alleles: one dog was heterozygous with product lengths of 262 and 264 bp and six dogs were homozygous for the 262 bp fragment.

## *Htr1A* genotyping in seven dog breeds

We determined the genotypes of the *htr1A* SNPs in eleven Golden Retrievers, five Beagles, seven Boxers, three Cairn Terriers, six Dobermanns, five Norwegian Elkhounds, and four Shetland Sheepdogs (Table 2). At least three SNP haplotypes were detected. Twenty-five dogs were homozygous for SNP haplotype T-C and five Boxers were homozygous for the haplotype G-A. A third SNP haplotype (G-C) was identified in three Norwegian Elkhounds, which were heterozygous at position 65 and homozygous C at position 808. Eight dogs (one Golden Retriever, two Beagles, one Cairn Terrier, and four Dobermanns) were heterozygous at both loci. Their haplotypes could therefore not be determined with certainty. We also genotyped the marker UU160O12 in

```
DOG      -------MET TALNSQKELS ACKDKEDCQE NGVLQQG---  30
HUMAN    -------MET TPLNSQKQLS ACEDGEDCQE NGVLQKV---  30
COW      -------MET TPLNSQKELS AYKDGEDCQE NGVLQKG---  30
MOUSE    -------MET TPLNSQKVLS ECKDKEDCQE NGVLQKG---  30
RAT      -------MET TPLNSQKVLS ECKDREDCQE NGVLQKG---  30
CHICKEN  MENKATSNET QPLTSKKGIS DCNEGEDCKE NGLLIRNPKS  40

DOG      ---------- -VPVPEDKVE SGQISSGYSA VPSPG-----  54
HUMAN    ---------- -VPTPGDKVE SGQISNGYSA VPSPG-----  54
COW      ---------- -VPAPGDKAE SGQISNGYSA VPNPG-----  54
MOUSE    ---------- -VPTPADKAE PGQISNGYSA VPSTS-----  54
RAT      ---------- -VPTTADRAE PSQISNGYSA VPSTS-----  54
CHICKEN  ALRLVDDGNK VHPGQGDKEE AAQISNGYSG VQSSGPCSGM  80

DOG      -AGDDPQHSI PAATTALVAE VHQ------- ------GERE  80
HUMAN    -AGDDTRHSI PATTTTLVAE LHQ------- ------GERE  80
COW      -AGDDTQHSI PAATTALVAE VHP------- ------AERE  80
MOUSE    -AGDEAPHST PAATTTLVAE IHQ------- ------GERE  80
RAT      -AGDEASHSI PAATTTLVAE IRQ------- ------GERE  80
CHICKEN  GEAEDAQCTA PAATTTTTTT TSTTCGAEGQ QQLMELGDRE 120

DOG      AWGKKMDFLL SVIGYAVDLG NVWRFPYICY QNGGGAFLLP 120
HUMAN    TWGKKVDFLL SVIGYAVDLG NVWRFPYICY QNGGGAFLLP 120
COW      TWAKKVDFLL SVIGYAVDLG NVWRFPYICY QNGGGAFLLP 120
MOUSE    TWGKKMDFLL SVIGYAVDLG NIWRFPYICY QNGGGAFLLP 120
RAT      TWGKKMDFLL SVIGYAVDLG NIWRFPYICY QNGGGAFLLP 120
CHICKEN  TWSKKIDFLL SVIGYAVDLG NVWRFPYICY QNGGGAFLIP 160

DOG      YTIMAIFGGI PLFYMELALG QYHRNGCISI WRKICPIFKG 160
HUMAN    YTIMAIFGGI PLFYMELALG QYHRNGCISI WRKICPIFKG 160
COW      YTIMAIFGGI PLFYMELALG QYHRNGCISI WTKICPIFKG 160
MOUSE    YTIMAIFGGI PLFYMELALG QYHRNGCISI WRKICPIFKG 160
RAT      YTIMAIFGGI PLFYMELALG QYHRNGCISI WRKICPIFKG 160
CHICKEN  YTIMAIFGGI PLFYMELALG QYHRNGCISI WRKICPIFKG 200

DOG      IGYAICIIAF YIASYYNTIM AWALYYLISS FTDQLPWTSC 200
HUMAN    IGYAICIIAF YIASYYNTIM AWALYYLISS FTDQLPWTSC 200
COW      IGCAICLIAF YIASYYNTII AWALYYLISS FTEQLPWTSC 200
MOUSE    IGYAICIIAF YIASYYNTII AWALYYLISS FTDQLQWTSC 200
RAT      IGYAICIIAF YIASYYNTII AWALYYLISS LTDRLPWTSC 200
CHICKEN  IGFAICIIDL YVASYYNTIM AWVFYYLVSS FTTELPWTSC 240

DOG      KNSWNTGNCT NYFSGDNITW TPHSTSPAEE FYMRHVLQLH 240
HUMAN    KNSWNTGNCT NYFSEDNITW TLHSTSPAEE FYTRHVLQIH 240
COW      ENSWNTGNCT NYFSEDNITW TLHSTSPAEE FYTRHVLQIH 240
MOUSE    KNSWNTGNCT NYFAQDNITW TLHSTSPAEE FYLRHVLQIH 240
RAT      TNSWNTGNCT NYFAQDNITW TLHSTSPAEE FYLRHVLQIH 240
CHICKEN  NNAWNTGNCT TYFSKDNISW ALHSISPAEE FYTRQVLQVH 280

DOG      RSNGLQDLGG ISWQLTLCIM LIFVVIYFSI WKGVKTSGKV 280
HUMAN    RSKGLQDLGG ISWQLALCIM LIFTVIYFSI WKGVKTSGKV 280
COW      RSKGLQDLGG LSWQLVLCIM FIFTVIYFSI WKGVKTSGKV 280
MOUSE    QSKGLQDLGT ISWQLALCIM LIFTIIYFSI WKGVKTSGKV 280
RAT      QSKGLQDLGT ISWQLTLCIV LIFTVIYFSI WKGVKTSGKV 280
CHICKEN  RSNGLDDLGG ISWQLTLCLL LIFIIVYFSI WKGVKTSGKV 320

DOG      VWVTATFPYI ILSVLLVRGA TLPGAWRGVL FYLKPNWQKL 320
HUMAN    VWVTATFPYI ILSVLLVRGA TLPGAWRGVL FYLKPNWQKL 320
COW      VWVTATFPYI ILLILLVRGA TLPGAWRGVL FYLKPNWQKL 320
MOUSE    VWVTATFPYI VLSVLLVRGA TLPGAWRGVV FYLKPNWQKL 320
RAT      VWVTATFPYI VLSVLLVRGA TLPGAWRGVV FYLKPNWQKL 320
CHICKEN  VWVTATFPYV ILFILLVRGA TLPGAWRGVL YYLKPEWQKL 360

DOG      LETGVWVDAA AQIFFSLGPG FGVLLAFASY NKFNNNCYQD 360
HUMAN    LETGVWIDAA AQIFFSLGPG FGVLLAFASY NKFNNNCYQD 360
COW      LETGVWVDAA AQIFFSLGPG FGVLLAFASY NKFHNNCYQD 360
MOUSE    LETGVWVDAA AQIFFSLGPG FGVLLAFASY NKFNNNCYQD 360
RAT      LETGVWVDAA AQIFFSLGPG FGVLLAFASY NKFNNNCYQD 360
CHICKEN  LATEVWVDAA AQIFFSLGPG FGVLLAYASY NKFHNNCYQD 400
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| Species | | | | | |
|---|---|---|---|---|---|
| DOG | ALVTSVVNCM | TSFVSGFVIF | TVLGYMAEMR | NEDVSEVAKD | 400 |
| HUMAN | ALVTSVVNCM | TSFVSGFVIF | TVLGYMAEMR | NEDVSEVAKD | 400 |
| COW | ALVTSAVNCM | TSFVSGFVIF | TVLGYMAEMR | KEDVSEVAKD | 400 |
| MOUSE | ALVTSVVNCM | TSFVSGFVIF | TVLGYMAEMR | NEDVSEVAKD | 400 |
| RAT | ALVTSVVNCM | TSFVSGFVIF | TVLGYMAEMR | NEDVSEVAKD | 400 |
| CHICKEN | ALVTSTVNCL | TSFVSGFVIF | TVLGYMAEMR | NEDVSEVAKD | 440 |

| Species | | | | | |
|---|---|---|---|---|---|
| DOG | AGPSLLFITY | AEAIANMPAS | TFFAIIFFLM | LITLGLDSTF | 440 |
| HUMAN | AGPSLLFITY | AEAIANMPAS | TFFAIIFFLM | LITLGLDSTF | 440 |
| COW | AGPSLLFITY | AEAIANMPAS | TFFAIVFFLM | LITLGLDSTF | 440 |
| MOUSE | AGPSLLFITY | AEAIRNMPAS | TFFAIIFFLM | LITLGLDSTF | 440 |
| RAT | AGPSLLFITY | AEAIANMPAS | TFFAIIFFLM | LITLGLDSTF | 440 |
| CHICKEN | MGPSLLFITY | AEAIANMPAS | TFFAIIFFLM | LLTLGLDSTF | 480 |

| Species | | | | | |
|---|---|---|---|---|---|
| DOG | AGLEGVITAV | LDEFPHIWSK | RREWFVLCVV | ITCFFGSLVT | 480 |
| HUMAN | AGLEGVITAV | LDEFPHVWAK | RRERFVLAVV | ITCFFGSLVT | 480 |
| COW | AGLEGVITAV | LDEFPHVWAK | RREWFVLGVV | ITCFFGSLVT | 480 |
| MOUSE | AGLEGVITAV | LDEFPHIWAK | RREWFVLIVV | ITCILGSLLT | 480 |
| RAT | AGLEGVITAV | LDEFPHIWAK | RREWFVLIVV | ITCVLGSLLT | 480 |
| CHICKEN | AGLEGVITGV | LDEFPHVWSK | RREFFVLGLI | IICFLGSLAT | 520 |

| Species | | | | | |
|---|---|---|---|---|---|
| DOG | LTFGGAYVVK | LLEEYATGPA | VLTVALIEAV | AVYWFYGITQ | 520 |
| HUMAN | LTFGGAYVVK | LLEEYATGPA | VLTVALIEAV | AVSWFYGITQ | 520 |
| COW | LTFGGAYVVK | LLEEFATGPA | VLTVALIEAV | AVFWFYGINQ | 520 |
| MOUSE | LTSGGAYVVT | LLEEYATGPA | VLTVALIEAV | VVSWFYGITQ | 520 |
| RAT | LTSGGAYVVT | LLEEYATGPA | VLTVALIEAV | AVSWFYGITQ | 520 |
| CHICKEN | LTFGGAYVVK | LFEEYATGPA | VLTVVFLEAV | AVAWFYGITQ | 560 |

| Species | | | | | |
|---|---|---|---|---|---|
| DOG | FCSDVKEMLG | FSPGWFWRIC | WVAISPLFLL | FIICSFLMSP | 560 |
| HUMAN | FCRDVKEMLG | FSPGWFWRIC | WVAISPLFLL | FIICSFLMSP | 560 |
| COW | FCSDVKEMLG | FSPGWFWKIC | WVAISPLFLL | FIICSFLMSP | 560 |
| MOUSE | FCSDVKEMLG | FSPGWFWRIC | WVAISPLFLL | FIICSFLMSP | 560 |
| RAT | FCSDVKEMLG | FSPGWFWRIC | WVAISPLFLL | FIICSFLMSP | 560 |
| CHICKEN | FCNDVKEMLG | FAPGWYWRVC | WVAISPIFLL | FVTCSFLSNP | 600 |

| Species | | | | | |
|---|---|---|---|---|---|
| DOG | PQLRLFQYNY | PQWSIILGYC | IGTSSFICIP | TYITYRLVTT | 600 |
| HUMAN | PQLRLFQYNY | PYWSIILGYC | IGTSSFICIP | TYIAYRLIIT | 600 |
| COW | PQLRLFQYDY | PRWSIILGYC | IGTSSFICIP | TYITYRLIVT | 600 |
| MOUSE | PQLRLFQYNY | PHWSIILGYC | IGTSSVICIP | IYIIYRLIST | 600 |
| RAT | PQLRLFQYNY | PHWSIVLGYC | IGMSSVICIP | TYIIYRLIST | 600 |
| CHICKEN | PELRLFDYNY | PYWTTVVGYC | IGTSSIICIP | IYMAYRLIIT | 640 |

| Species | | | | |
|---|---|---|---|---|
| DOG | PGTFKERIIR | GITPETATEI | PYGDIHLNAI | 630 |
| HUMAN | PGTFKERIIK | SITPETPTEI | PCGDIRLNAV | 630 |
| COW | PGTLKERIIK | GITPETPTAI | PCGDIRLNAV | 630 |
| MOUSE | PGTLKERIIK | SITPETPTEI | PCGDIRMNAV | 630 |
| RAT | PGTLKERIIK | SITPETPTEI | PCGDIRMNAV | 630 |
| CHICKEN | PGTLKERILK | SITPETATEI | PFGDIRMNAV | 670 |

**Figure 3.** Alignment of the amino acid sequence of the canine serotonin transporter with human (accession number NP_001036), bovine (NP_777034), murine (NP_034614), rat (CAA71909), and chicken (NP_998737) sequences. Amino acids are presented in groups of ten. Residues that are conserved in the six species are shown in grey background and predicted transmembrane regions according to SWISSPROT (http://us.expasy.org/cgi-bin/niceprot.pl?P31645) are boxed. Residues 1-87 form the amino terminus; residues 109-115 form the first extracellular loop, and so on. The following key residues are underlined: 95Y and 586F are antagonist binding sites; 98D is a coordination site for 5-HT; 172I and 176Y are binding sites for 5-HT; 179I is part of an external gate; 200C and 209C form a disulfide bridge; 208N and 217N are N-linked glycosylation sites; and 8S, 13S, 277S, and 603T are potential sites of protein kinase A and C phosphorylation. All positions of key residues refer to the canine protein.

these dogs and found six alleles with product lengths of 293, 295, 297, 299, 303, and 305 bp.

**Table 2.** Genotype frequencies of two single nucleotide polymorphisms (SNPs) in *htr1A* in 41 dogs of diverse breeds.

| | n | SNP 65 genotype | | | SNP 808 genotype | | |
|---|---|---|---|---|---|---|---|
| | | **TT** | **TG** | **GG** | **CC** | **CA** | **AA** |
| **Golden Retriever** | 11 | 10 | 1 | 0 | 10 | 1 | 0 |
| **Beagle** | 5 | 3 | 2 | 0 | 3 | 2 | 0 |
| **Boxer** | 7 | 2 | 0 | 5 | 2 | 0 | 5 |
| **Cairn Terrier** | 3 | 2 | 1 | 0 | 2 | 1 | 0 |
| **Dobermann** | 6 | 2 | 4 | 0 | 2 | 4 | 0 |
| **Norwegian Elkhound** | 5 | 2 | 3 | 0 | 5 | 0 | 0 |
| **Shelti** | 4 | 4 | 0 | 0 | 4 | 0 | 0 |

## Discussion

We characterised three canine serotonergic genes, isolated canine BAC clones containing them, developed oligonucleotides for genomic sequencing of their coding regions and intron-exon boundaries, and identified both single nucleotide polymorphisms and polymorphic microsatellite markers in or about the vicinity of the genes. This work provides a starting point for mutation scans and association studies on canine behavioural traits.

The release of the 7.8x redundant Boxer genome sequence and the 1.5x Poodle sequence has enabled a rapid elucidation of the structure of candidate genes in the dog (Kirkness *et al.* 2003; Sutter and Ostrander 2004). We retrieved canine DNA sequence contigs from chromosomes CFA02, CFA22, and CFA09 respectively for *htr1A*, *htr2A*, and *slc6A4*. The chromosomal location of these contigs is in accordance with the position of the genes on the human genome and with our previous work. We predicted *htr1A* to be located on CFA02 by radiation hybrid mapping (section 3.1). *HTR2A* maps to human chromosome 13q14-21, which displays synteny with CFA22. A large section of CFA09 corresponds to HSA17, which contains human *SLC6A4* (Guyon *et al.* 2003).

## Genotyping in Golden Retrievers

This study is embedded in a research project involving aggressive behaviour in Golden Retrievers and we analysed the coding sequence of the three genes in dogs of this breed. The Golden Retriever breed has been shown to be relatively heterogeneous (Nielen *et al.* 2001; Sutter *et al.* 2004), which is in agreement with the large number of haplotypes that we describe here. The SNPs that were identified in *htr2A* and the intron of *slc6A4* in this study are close to splice sites and may affect splicing (Pagani and Baralle 2004). Alternative splicing of the serotonin receptor 2A has been demonstrated in human brain cDNA (Guest *et al.* 2000).

## ***Htr1A*** **genotyping in seven dog breeds**

The domestication of dogs is likely to have involved genetic selection for less fearful and aggressive behaviour in wolf ancestors. *Htr1A* is a strong candidate for involvement in this process because indications suggest that it plays a role in anxious behaviour. If *htr1A* were indeed involved in domestication, we would expect to find little variation in this region of the canine genome. We have analysed the genotypes of *htr1A* in dogs from seven breeds and have identified at least three SNP haplotypes. This result does not support involvement of the gene in the domestication of the wolf. However, this conclusion has to be treated with caution because patterns of polymorphism cannot always be used in the search for domestication genes. The amount by which variation is reduced by strong artificial selection depends on the initial frequency of the beneficial allele (Innan and Kim 2004).

Although the SNPs in canine *htr1A* are not likely to affect the function of the receptor, it is possible that they are in linkage disequilibrium with functional polymorphisms in a regulatory region of the gene. Differences in *htr1A* haplotype frequencies between dog breeds with diverse behavioural characteristics might point to an influence of *htr1A* on canine temperament. The *htr1A* haplotype frequencies in the group of Boxers seemed to differ from those in other breeds. However, our study group is too small to draw firm conclusions on this topic. It will be very interesting to study breed differences in *htr1A* haplotype frequencies in more detail, as was already done for the gene encoding the serotonin receptor 1B (*htr1B*) by Masuda *et al.* (2004b). These researchers detected interbreed-variations in genotype and allele frequencies of *htr1B* SNPs.

## Acknowledgements

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# 3.4
# Evaluation of four genes involved in serotonin binding and transport as candidates for aggression in Golden Retrievers

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Bernard A. van Oost, and Peter A.J. Leegwater

This section has been submitted for publication.

## Abstract

Alterations in brain serotonin metabolism have been described in aggressive dogs. Here, we evaluate four genes of the canine serotonergic system (serotonin receptor 1A, 1B and 2A gene and the serotonin transporter gene) as candidates for aggression in Golden Retriever dogs. We performed mutation screens and linkage analysis. The coding DNA sequence of the genes was identical in aggressive and non-aggressive dogs, apart from single nucleotide polymorphisms that were previously reported. An affecteds-only parametric linkage analysis revealed no linkage between the candidate genes and affection status. We conclude that it is unlikely that these genes play a major role in the variation in aggressive behaviour in Golden Retrievers.

## Introduction

Dogs have been living in close proximity to humans since the last Ice Age (Clutton-Brock 1995). Like their progenitor the grey wolf, dogs respond with aggressive behaviour to certain stimuli. This is natural behaviour in the majority of cases (Borchelt and Voith 1996; Mugford 1984; Reisner 1997). However, canine aggression can develop into a dangerous problem (Winkler 1977; Wright 1985). There is individual variation in the tendency of dogs to display aggressive behaviour. This variation is the result of a complex system of interacting genes and environmental influences, which is poorly understood.

We study the genetics of aggression in families of Golden Retriever dogs. Golden Retrievers are reputably friendly pets. However, there are reports of very aggressive Golden Retrievers (Edwards 1991; Galac and Knol 1997; Heath 1991; Knol and Schilder 1999). We found a high heritability of human-directed aggression in a group of 325 Golden Retrievers, suggesting possible involvement of a major gene (section 2.3). It is more feasible to map genes that influence behavioural traits in dog families than in human families (Sutter and Ostrander 2004). This is because dog breeds are genetic isolates. The low genetic heterogeneity within dog breeds implies that only a limited number of the genes that influence a certain behavioural trait will be functionally polymorphic within a breed. This greatly facilitates the chances of finding such genes. In the present paper, we evaluate four genes of the serotonergic system as candidates for involvement in aggression in Golden Retrievers.

The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) plays a central role in the molecular biology of the mind. The influence of 5-HT on normal and abnormal behaviour has been studied extensively (reviewed by Gingrich and Hen 2001; Lesch and Merschdorf 2000; Lucki 1998). Abnormalities in 5-HT metabolism have been found in humans with pathological aggression and anxiety (Berman and Coccaro 1998). There is evidence for a role of the 5-HT system in canine aggression as well. For instance, Reisner and colleagues (1996) reported decreased levels of 5-hydroxyindoleacetic acid (the major metabolite of 5-HT) in cerebrospinal fluid of dominant aggressive dogs. Badino *et al.* (2004) found modifications of 5-HT receptor concentrations in brains of aggressive dogs.

Four genes encoding components of the serotonergic system are particularly good candidates for the regulation of aggressive behaviour. Figure 1 is a schematic representation of the role of the gene products in 5-HT neurotransmission. The first candidate gene (*htr1A*) encodes serotonin receptor 1A. Serotonin receptor 1A seems to play a role in anxiety, depression, stress response, and aggression. *Htr1A* knockout mice show increased anxiety

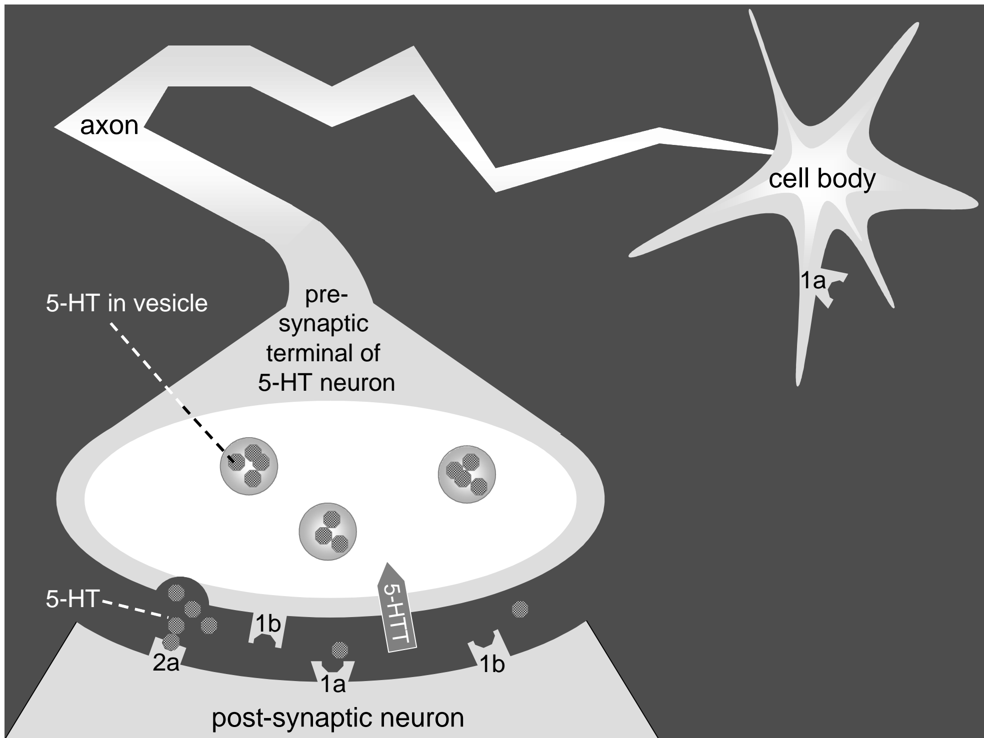


**Figure 1.** Schematic view of the role of the four candidate genes in central serotonergic (5-HT) neurotransmission. Serotonin receptors 1A (1a in the figure) are localised on serotonergic cell bodies and dendrites in the raphe nuclei of the brain, where they act as autoreceptors to inhibit cell firing. A number of limbic structures contain postsynaptic $5\text{-HT}_{1A}$ receptors. Serotonin receptors 1B (1b in the figure) can be found both pre- and postsynaptically on neurons in several brain areas. They modulate the release of 5-HT and other neurotransmitters. Serotonin receptors 2A (2a in the figure) are widely distributed in peripheral and central tissues. They are located postsynaptically. The serotonin transporter (5-HTT in the figure) is localised on axon terminals and cell bodies and dendrites of 5-HT neurons. The 5-HTT is responsible for the reuptake of 5-HT from brain synapses. (See Barnes and Sharp 1999; Feldman *et al.* 1997; Gingrich and Hen 2001; Hoyer *et al.* 1994 or 2002; Vergé and Calas 2000 for more detailed discussions of the genes.)

(Heisler *et al.* 1998; Parks *et al.* 1998; Ramboz *et al.* 1998). Knockout mice lacking the second candidate gene, the gene encoding serotonin receptor 1B (*htr1B*), display increased aggression (Saudou *et al.* 1994). The third candidate gene is the gene encoding serotonin receptor 2A (*htr2A*). A mutation in the human *HTR2A* gene is associated with altered 5-HT binding. This altered 5-HT binding has been implicated in schizophrenia, suicidal behaviour, impaired impulse control, and aggression history (Abdolmaleky *et al.* 2004; Bjork *et al.*

2002; Khait *et al.* 2005). Peremans and colleagues (2003) found an increased binding index of serotonin 2A receptors in cortical brain regions of impulsive aggressive dogs. The fourth candidate is the gene encoding the serotonin transporter (*slc6A4*). A polymorphism in its promoter region influences serotonin transporter density in the brain and is associated with mental disorders in humans (Anguelova *et al.* 2003; Hariri *et al.* 2002; Katsuragi *et al.* 1999; Lesch *et al.* 1996; Lesch *et al.* 1999). *Slc6A4* knockout mice show reduced aggression (Holmes *et al.* 2003).

We have described the isolation and characterization of these genes in the dog in section 3.1, 3.2, and 3.3. In this section, we evaluate the genes as candidates for aggression in Golden Retrievers. We performed mutation screens of the coding DNA sequence in unrelated aggressive Golden Retrievers. In addition, we used linkage analysis to determine the likelihood of the presence of a “major aggression locus” in or close to the genes.

## Materials and methods

### Animals and DNA isolation

All dogs were privately owned Golden Retrievers. Some of the dogs were referred to our clinic because of their aggressive behaviour (probands). We recruited the others because they were related to one of 36 probands. As is usual in dog breeds, all probands were related to each other within a limited number of generations. For linkage analysis, small families consisting of probands and their close relatives (siblings, half-siblings, parents, grandparents and cousins) were extended by merging families or adding single probands if there were not more than three meioses separating aggressive dogs. In this way we created 23 families. Thirteen of the 23 families contained only one aggressive dog. Such families are not informative in an affecteds-only analysis, so they were excluded from further analysis. The remaining were used for linkage analysis; their pedigrees are depicted in the Appendix. DNA samples were available for 98 individuals in these families (33 affecteds and 65 unaffecteds). Genomic DNA was isolated from whole blood leucocytes using a standard protocol (Miller *et al.* 1988).

### Phenotyping

We have collected various quantitative measures of aggressiveness for the dogs (section 2). One of these measures was the impression of the dog owner.

When a dog was included in the study, its owner was asked for his or her opinion about the aggressiveness of the dog in a personal interview. We asked the owners if their dog was aggressive towards humans and if it was aggressive towards other dogs. The status of the dog was coded in three classes: non-aggressive (score 1), threatens (score 2), or bites (score 3). We used these owner impressions in the present study because they were available for all dogs and because the results of our quantitative genetic analyses suggest that they are suitable for genetic studies (section 2.3).

**Mutation screening of the coding DNA sequence**

We analysed the coding DNA sequence (CDS) of the four candidate genes in seven (*htr1A* and *htr1B*) or eight (*htr2A* and *slc6A4*) probands. The probands were selected for high aggression scores. For *htr1A* and *htr1B* we used two dogs that had bitten both humans and other dogs, one dog that had bitten humans and threatened other dogs, two dogs that had bitten only humans, and two dogs that had bitten only other dogs. For *htr2A* and *slc6A4*, we used two dogs that had bitten both humans and other dogs, one dog that had bitten humans and threatened other dogs, two dogs that had only bitten humans, one dog that had only bitten other dogs, and one dog that had threatened people. The probands had no known common grandparents. The CDS was amplified and sequenced using overlapping primer pairs as described in section 3.2 and 3.3. Possible functional effects of polymorphisms were predicted with POLYPHEN (http://genetics.bwh.harvard.edu/cgi-bin/pph/polyphen.cgi). Effects of polymorphisms close to splice sites were predicted with three splice prediction programs: NetGene2 (Brunak *et al.* 1991), Splice Prediction by Neural Network (Reese *et al.* 1997), and SpliceSiteFinder (Shapiro and Senapathy 1987). Analysis of the CDS of the candidate genes in non-aggressive Golden Retrievers has been described in section 3.2 and 3.3 For *htr1A* and *htr1B* these non-aggressive dogs were unrelated to the probands. For *htr2A* and *slc6A4* we used one non-aggressive sibling of each proband.

**Selection and genotyping of markers for linkage analysis**

We have described the identification of polymorphic markers for the candidate genes in section 3.1, 3.2, and 3.3 of this thesis. This work resulted in three markers for *htr1A* (single nucleotide polymorphisms (SNPs) G65T and A808C and microsatellite marker UU160O12), six markers for *htr1B* (SNPs A157C, G246A, C660G, T955C, and G1146C and microsatellite marker UU18L8), two markers for *htr2A* (a SNP at intervening sequence (IVS) 2-10 and microsatellite

marker UUHTR2AEX2), and three markers for *slc6A4* (SNPs C411T and IVS9-12 and microsatellite marker UU76G6). Masuda *et al.* (2004b) mentioned a sixth SNP in the *htr1B* CDS: G57A. We genotyped the markers in a small group of Golden Retrievers in order to evaluate their informativity. Based on these results, we selected three microsatellite markers and seven single nucleotide polymorphisms for linkage analysis (Table 1).

Microsatellite markers were genotyped after PCR on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). PCR conditions are described in section 3.2 and 3.3. GENESCAN 3.7 software was used for genotype assessment. Single nucleotide polymorphism genotyping was performed by DNA sequencing of PCR products on the ABI 3100 Genetic Analyzer (section 3.2 and 3.3). The DNA sequence chromatograms were inspected using LASERGENE software in order to detect heterozygotes. We combined several markers into haplotypes for the genes *htr1B*, *htr2A*, and *slc6A4*. Haplotypes were deduced with certainty for dogs that were heterozygous for not more than one marker. For dogs that were heterozygous for multiple markers, we deduced the haplotypes from the data of relatives. If this was not possible, we assigned the most frequently observed haplotypes to the multiple heterozygous dogs.

**Linkage analysis**

We performed a parametric affecteds-only linkage analysis in order to determine whether the candidate gene haplotypes were linked to aggressive behaviour in the Golden Retrievers. The owner impressions were converted into a categorical variable for linkage analysis. Probands were classified as “affected” if they scored at least 2 (i.e. threatening) on human-directed aggression. Dogs recruited as a relative of a proband were only classified as “affected” if they scored 3 (biting) on human-directed aggression. All other dogs were classified as “unaffected”.

Marker haplotype frequencies were determined in a group of 27 (*htr1A* and *htr2A*), 31 (*htr1B*), or 26 (*slc6A4*) parent dogs. We either had a DNA sample of these dogs, or we could deduce their haplotypes from haplotypes of their children. This group also included dogs from the 13 families that were not included in the linkage analysis.

The mode of inheritance of the aggressive phenotype in our families is unclear. We therefore analysed the data under both an autosomal dominant and an autosomal recessive model. The penetrance was set at 0.01 for the “aggressive genotype”. In this way, affected dogs are assumed to have the aggression allele, while it is not important for the calculation whether

**Table 1.** Markers used for linkage analysis.

| **Gene** [1] | **Type of marker** [2] | **Position of marker** [3] | **Alleles observed (allele frequency)** [4] | **Haplotypes observed (haplotype frequency)** [4] |
|---|---|---|---|---|
| ***htr1A*** | (CA)$_n$ (UU160O12) | *7370 | 297 (0.5)<br>303 (0.5) | - |
| ***htr1B*** | A/C SNP [5] | 157 | A (0.58)<br>C (0.42) | 143-A-G-T-G (0.20)<br>143-C-A-T-G (0.42)<br>143-A-G-C-C (0.06)<br>139-A-G-C-C (0.24)<br>139-A-G-T-G (0.05)<br>147-A-G-T-G (0.03) |
| | G/A SNP | 246 | G (0.58)<br>A (0.42) | |
| | T/C SNP | 955 | T (0.69)<br>C (0.31) | |
| | G/C SNP | 1146 | G (0.69)<br>C (0.31) | |
| | (GA)$_n$ (UU18L8) | -68395 | 139 (0.29)<br>143 (0.68)<br>147 (0.03) | |
| ***htr2A*** | C/T SNP | IVS 2-10 | C (0.85)<br>T (0.15) | 128-C (0.07)<br>130-C (0.41)<br>132-C (0.37)<br>132-T (0.15) |
| | (CA)$_n$ (UUHTR2AEX2) | IVS2+1439 | 128 (0.07)<br>130 (0.41)<br>132 (0.52) | |
| ***slc6A4*** | C/T SNP | 411 | C (0.75)<br>T (0.25) | C-G (0.75)<br>T-A (0.25) |
| | G/A SNP | IVS9-12 | G (0.75)<br>A (0.25) | |

[1] *htr1A, htr1B, htr2A* = respectively serotonin receptor 1A, 1B, and 2A gene; *slc6A4* = serotonin transporter gene
[2] SNP = single nucleotide polymorphism. Names of microsatellite markers have been included in brackets.
[3] Position refers to the coding sequence of the canine gene. We used the nomenclature recommended by den Dunnen and Antonarakis (2001): the A of the ATG start codon is designated number 1; the nucleotide 5´ to this A is numbered –1; and the nucleotide 3´ of the translation termination codon is *1. Positions in introns refer to the nearest exon. The nomenclature of the introns is based on the human gene structure. IVS = intervening sequence
[4] Allele and haplotype frequencies were determined in a group of 27 (*htr1A* and *htr2A*), 31 (*htr1B*), or 26 (*slc6A4*) parental animals.
[5] This polymorphism is nonsynonymous.

unaffected dogs lack the aggression allele. In other words, the software will only calculate likelihoods that aggressive dogs share alleles by descent from a common ancestor. The true penetrance of the aggressive phenotype is unknown. We assumed that there were no phenocopies in the families. We also assumed genetic homogeneity because all probands were related to each other. The frequency of the aggression allele was set at 0.1 to allow for multiple transmitting ancestors in the pedigrees. SUPERLINK software was used to calculate two-point logarithm of the odds (LOD) scores. This software is specifically suitable for analysis of pedigrees with many inbreeding loops (Fishelson and Geiger 2002; 2004).

In order to estimate the power of the pedigrees, we calculated the maximum obtainable LOD scores. Affected individuals were assigned haplotypes 2/2 in these calculations; unaffected parents were assigned haplotypes 1/2; and other unaffected individuals were assigned haplotypes 1/1. We assigned haplotypes 0/0 (unknown) to dogs from which we did not have a DNA sample. We assumed that there were four alleles of the hypothetical marker with equal allele frequencies.

## Results

### Mutation screening of the coding DNA sequence

The CDS of each candidate gene was scanned for mutations in seven (*htr1A* and *htr1B*) or eight (*htr2A* and *slc6A4*) aggressive Golden Retrievers. Analysis of the CDS in three (*htr1A*), six (*htr1B*), or eight (*htr2A* and *slc6A4*) non-aggressive Golden Retrievers has been described in section 3.2 and 3.3. There was no variation in the CDS of *htr1A* and *htr2A* in the Golden Retrievers. We observed five SNPs in the CDS of *htr1B* and one SNP in the CDS of *slc6A4*, which is in agreement with the results in section 3.2 and 3.3. The allele distribution of these SNPs in the two groups of Golden Retrievers did not indicate a role in aggressive behaviour. In conclusion, there seems to be no systematic difference between the CDS of the candidate genes in aggressive and non-aggressive Golden Retrievers.

### Selection and genotyping of markers for linkage analysis

The majority of the markers that we described in sections 3.1, 3.2, and 3.3 were polymorphic in the Golden Retrievers. Three markers displayed little variation in the Golden Retrievers: the *htr1A* SNPs G65T and A808C, and the *slc6A4*

microsatellite marker UU76G6. We have genotyped the *htr1A* SNPs in 38 Golden Retrievers (data not shown). Only two G-A haplotypes were observed in this group; 36 dogs were homozygous for the T-C haplotype. *Slc6A4* marker UU76G6 was genotyped in 15 dogs and only one dog was heterozygously 262/264 (data not shown). Because of their low informativity, these markers were excluded from the linkage analysis. For *htr1B* we used four out of five available SNPs in a region of only 990 bp. We did not observe the *htr1B* SNP G57A that was reported in Golden Retrievers by Masuda *et al.* (2004b). All of our Goldens were homozygously G at this position. Ten markers were included in the final linkage analysis (Table 1).

We observed two alleles for *htr1A* marker UU160O12 in both the parental group and the families that we used for linkage analysis (Table 1). The *htr1B* polymorphisms formed six haplotypes in the Goldens, three of which were rare (frequency<0.1). The SNPs A157C and G246A were in complete linkage disequilibrium. SNPs T955C and G1146C were also in complete linkage disequilibrium. We detected four haplotypes of *htr2A* in the Golden Retrievers. The two SNPs in *slc6A4* were in complete linkage disequilibrium and formed two haplotypes in the dogs. In the 10 families that we used for linkage analysis, haplotypes were deduced with certainty in 89% of the dogs for *htr1B*, 99% for *htr2A*, and 87% for *slc6A4*.

## Linkage analysis

We calculated the maximum achievable LOD score using hypothetical genotypes (see Materials and methods). The maximum LOD score generated by our pedigrees was 3.4 at recombination fraction θ=0 assuming a dominant mode of inheritance (Table 2). Under a recessive model, the maximum LOD score was 7.3 at θ=0. The families are therefore theoretically powerful enough to prove linkage.

There was no significant linkage between any of the candidate genes and the aggressive phenotype (Table 2). LOD scores

**Table 2.** Logarithm of the ODDS (LOD) scores.[1]

| Gene | Autosomal dominant | Autosomal recessive |
|---|---|---|
| ***htr1A*** | 0.33 | -0.15 |
| ***htr1B*** | -0.62 | -1.5 |
| ***htr2A*** | -1.4 | -2.9 |
| ***slc6A4*** | -0.064 | -0.51 |
| **maximum** | 3.4 | 7.3 |

[1] LOD scores were calculated with the following assumptions: frequency of the “aggression allele”=0.1; penetrance of the “aggression allele”=0.01; θ=0. Marker haplotype frequencies were deduced from a group of parental animals.

varied from -1.4 to +0.33 assuming dominant inheritance and from -2.9 to -0.15 assuming recessive inheritance. The highest LOD scores were obtained for *htr1A* (+0.33 under a dominant model and -0.15 under a recessive model). The lowest LOD scores were obtained for *htr2A* (-1.4 under a dominant model and -2.9 under a recessive model).

## Discussion

Neither the mutation screen, nor the linkage analysis provides evidence for a major role of the candidate genes in aggressive behaviour in the families of Golden Retrievers. We did not detect any mutations in the CDS of the genes specific for aggressive dogs. There were polymorphisms in the CDS of *htr1B* (A157C, G246A, C660G, T955C, G1146C) and *slc6A4* (C411T), but these are unlikely to have functional significance. All SNPs except A157C in *htr1B* were synonymous. The A157C variation, resulting in an isoleucine/leucine polymorphism of amino acid 53, was predicted to be functionally insignificant by POLYPHEN. The genes *htr2A* and *slc6A4* contain SNPs close to splice sites (at position IVS2-10 and IVS9-12, respectively) that could theoretically affect splicing. However, the polymorphisms did not have a large effect on splice site prediction by three programs (NetGene2, Splice Prediction by Neural Network, and SpliceSiteFinder). It is thus unlikely that the polymorphisms affect splicing.

The absence of mutations in the CDS does not exclude a candidate gene because there could be mutations in regulatory regions of the gene. Polymorphisms or mutations in regulatory regions such as the promoter may play an important role in behavioural variation. Such polymorphisms can affect gene expression and some scientists suggest that behavioural differences may result from variation in gene expression rather than structural variation in genes (Hamer 2002; Saetre *et al.* 2004). Mutations in regulatory regions can be detected through direct sequencing of the non-coding DNA sequence. However, these regions are not well defined for the genes at hand. We therefore used the alternative strategy of linkage analysis. The markers that we used for linkage analysis were located either in the candidate genes or very close to the genes. Mutations in non-coding regions of the gene are linked to the marker alleles in the analysed families. The LOD scores therefore represent the likelihood that there is an aggression locus in coding or non-coding regions of the genes.

A LOD score of 3 is usually considered as evidence for linkage, whereas LOD scores below -2 exclude the gene. Strictly speaking, we cannot

exclude any of our candidate genes. However, the LOD scores for *htr1B* and *htr2A* were quite low. A major role of these genes is highly unlikely. The results for *slc6A4* and *htr1A* are less conclusive. This is probably the result of the poor informativity of the markers. For both genes, we observed only two alleles or haplotypes with high frequencies. Typing of additional markers might help to definitively exclude the genes. However, in the light of the observed low level of variation, it is unlikely that *htr1A* and *slc6A4* play a major role in aggression in the Golden Retriever families. For a small number of individuals we did not obtain genotypes (genotype 0/0 in the pedigrees in the Appendix). We do not expect these individuals to have large effects on the LOD scores.

The study design that we used is probably not powerful enough to detect genes of small effect. We can therefore not rule out the possibility that variation in the candidate genes has a small effect on the variation in aggression. It may be preferable to use quantitative instead of qualitative linkage analysis in future studies. All available dogs (including those from the 13 families that were excluded here) can be included in a quantitative analysis.

In this paper, we have demonstrated that it is unlikely that the four candidate genes of the serotonergic system play a major role in the variation of aggression in our Golden Retriever families. A long list of other candidate genes could be generated, including for instance the gene encoding tryptophan hydroxylase 2 (Harvey *et al.* 2004; Walther *et al.* 2003; Zhang *et al.* 2004; Zill *et al.* 2004) and the gene encoding monoamine oxidase A (Brunner *et al.* 1993a and 1993b; Cases *et al.* 1995; Caspi *et al.* 2002). (See Miczek *et al.* 2001 for a review of aggression genes in mice.) However, with the completion of the dog genome project, genome-wide linkage and association studies in dogs have become feasible (Lindblad-Toh *et al.* 2005). In genome-wide analyses there is no *a priori* assumption about which genes are involved in the phenotypes. This opens the opportunity for finding genes that have not been associated with aggression up to date. Such studies are currently in progress.

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## Appendix: Pedigrees used for linkage analysis

This appendix consists of the pedigrees of 10 Golden Retriever families that were used for linkage analysis. Squares represent males; circles represent females. Black symbols represent aggressive dogs and white symbols represent non-aggressive dogs. (The method of phenotyping is explained in the Materials and methods paragraph.) We used grey symbols for dogs of which we have no behavioural information. Arrows indicate probands, i.e. dogs that were referred to our clinic because of their aggressive behaviour. Numbers and letters at the upper left corners of the symbols are identification numbers of the dogs. These numbers are in brackets if we have no DNA sample of the dog. The numbers beneath the symbols are the genotypes (*htr1A*) or haplotypes (*htr1B, htr2A,* and *slc6A4*) for the candidate genes (see Figure A1 and Table A1 for a legend to the symbols).

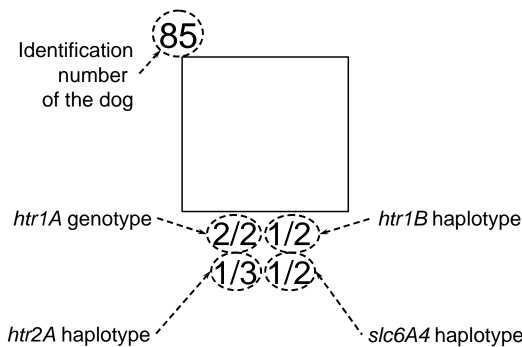
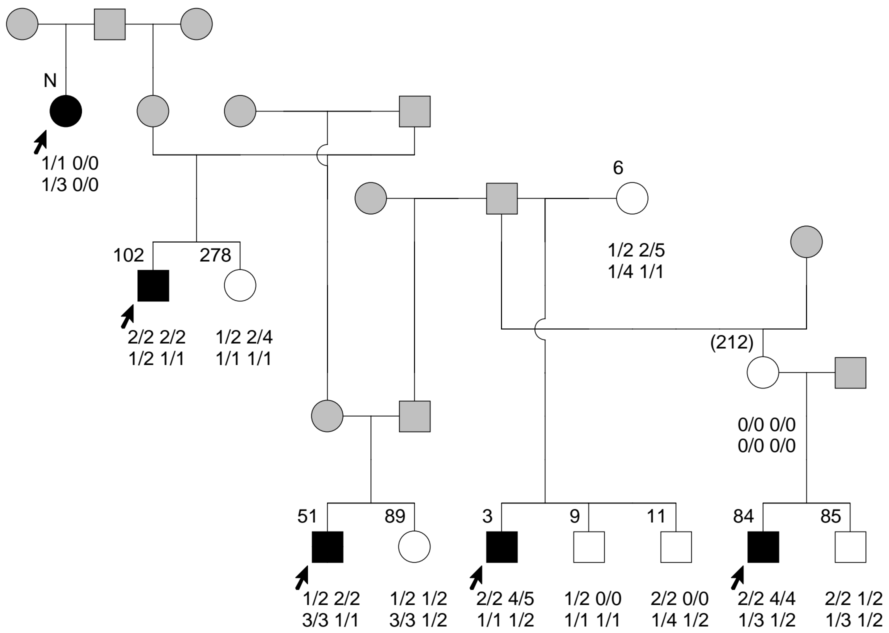



**Figure A1.** Legend to the symbols in the pedigrees of this appendix.

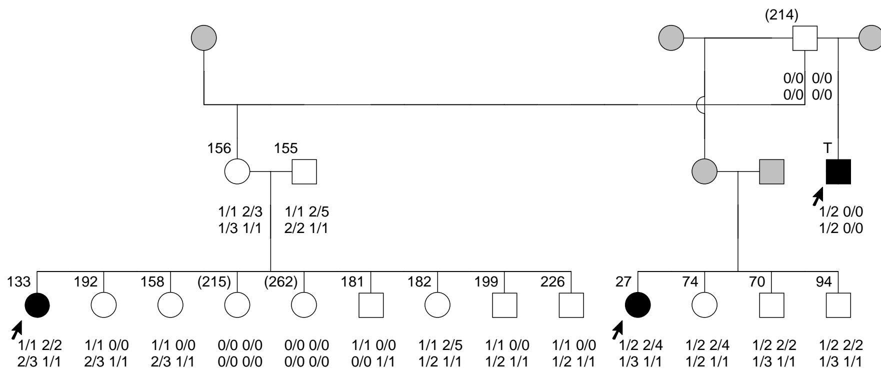
**Table A1.** Legend to the codes that are used for genotypes (*htr1A*) and haplotypes (*htr1B, htr2A,* and *slc6A4*) in the pedigrees of this appendix.

| | allele or haplotype | code in pedigrees |
|---|---|---|
| ***htr1A*** | unknown | 0 |
| | 297 | 1 |
| | 303 | 2 |
| ***htr1B*** | unknown | 0 |
| | 143-A-G-T-G | 1 |
| | 143-C-A-T-G | 2 |
| | 143-A-G-C-C | 3 |
| | 139-A-G-C-C | 4 |
| | 139-A-G-T-G | 5 |
| | 147-A-G-T-G | 6 |
| ***htr2A*** | unknown | 0 |
| | 132-C | 1 |
| | 130-C | 2 |
| | 132-T | 3 |
| | 128-C | 4 |
| ***slc6A4*** | C-G | 1 |
| | T-A | 2 |

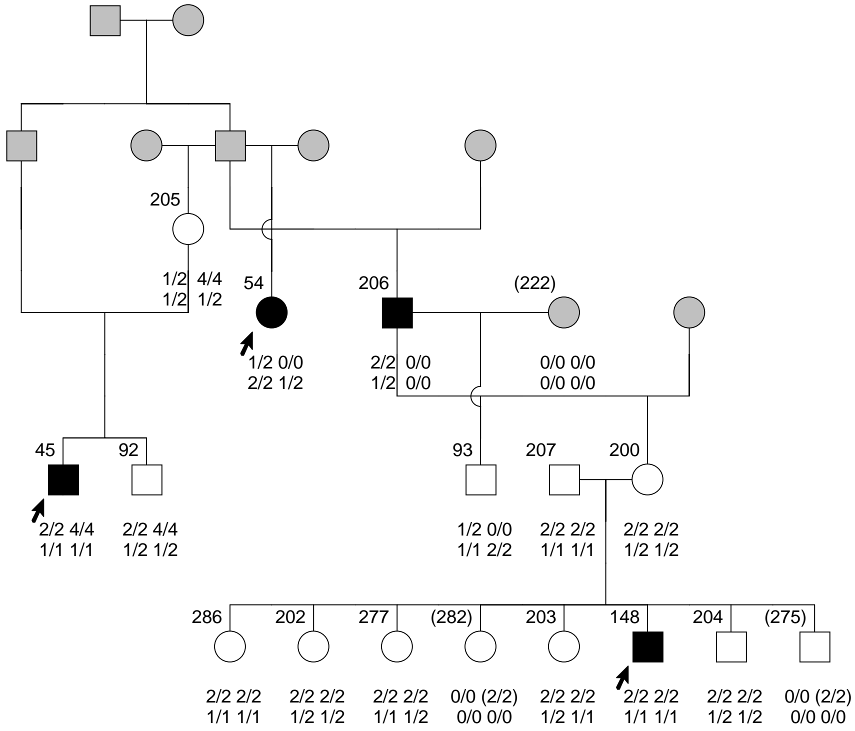
**Family 1**



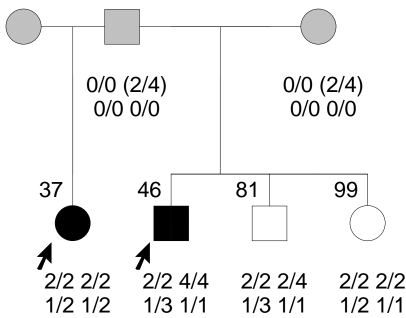

**Family 2**



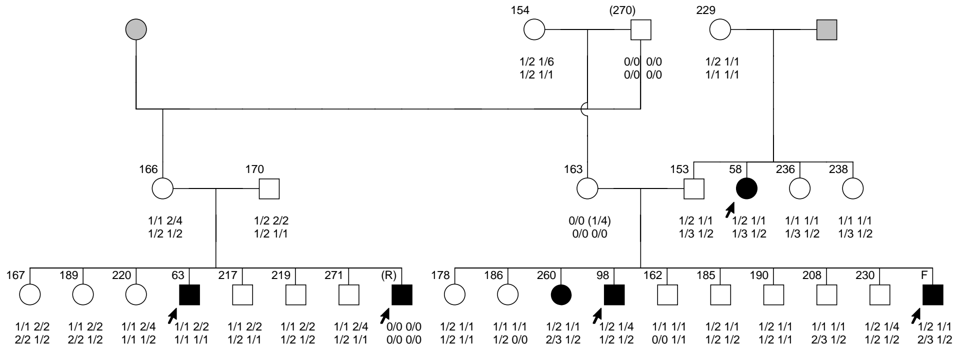
**Family 3**



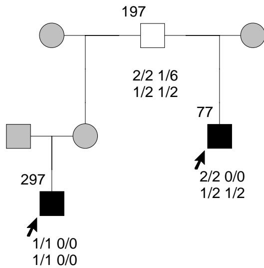

**Family 4**



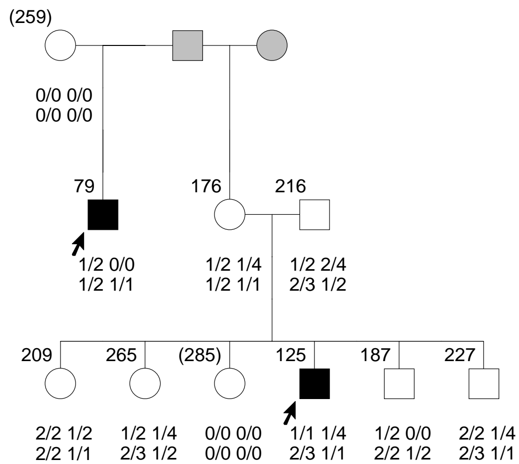
**Family 5**
(There are four meioses between dog 63 and the dogs in the sibship of 98. We made an exception to our "3-meioses-rule" because DNA samples from both parents were available for these two sibships.)



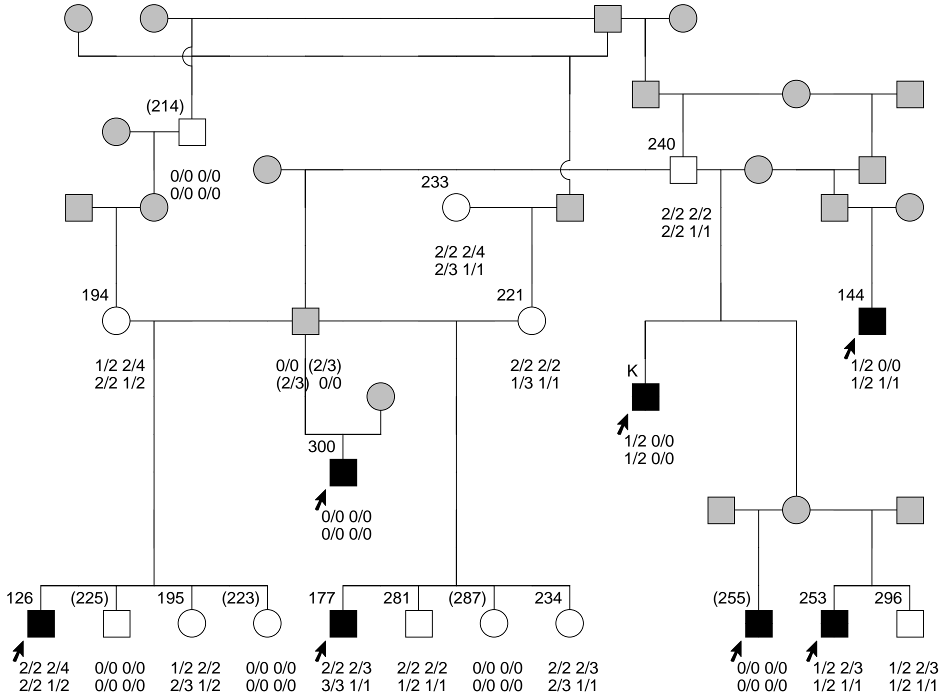

**Family 6**



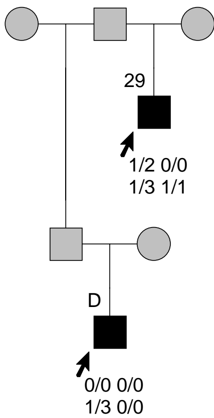

**Family 8**



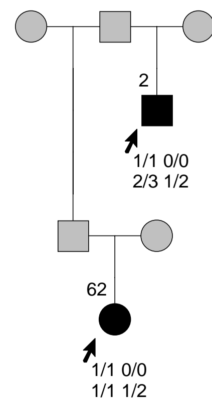
**Family 7**




**Family 9**




**Family 10**



CHAPTER 4

# General discussion

# 4
# General discussion

The aim of the work described in this thesis was to identify genetic variations underlying the variation in aggressive behaviour in Golden Retriever dogs. To achieve this aim, we performed extensive phenotype analysis, which is described in sections 2.1 and 2.2. We estimated the heritability of the various phenotypic measures in order to determine their usefulness for genetic studies (section 2.3). The results of the phenotype analysis were used in the molecular genetic studies of four serotonergic candidate genes in chapter 3. In this general discussion, we will discuss the results in more depth, place them in a broader perspective and discuss future plans.

## The evidence for a high heritability of aggression in Golden Retrievers

We started our studies of aggression in Golden Retrievers because early observations suggested a strong genetic basis for the behaviour. In other words, we expected that a large part of the phenotypic variation was caused by genetic variation. In retrospect, the evidence for a strong genetic basis was rather weak. The first indication was a subjective impression of a sudden increase in the number of aggressive Golden Retrievers referred to the Utrecht University Companion Animal Clinic. A high frequency of aggressive patients at a behavioural clinic is difficult to interpret, as we discussed in section 1.3. The second indication was the impression that the aggressive Golden Retrievers displayed a similar type of aggression (sometimes referred to as "Golden aggression" by breeders and veterinarians). Key elements were the unpredictability of the aggression, display of fearful behavioural elements by the dog (e.g. freezing and low posture), and approaching the dog as the trigger for the behaviour (personal communication with B.W. Knol). However, these are rather general characteristics that could be used to describe almost any aggressive dog. As we showed in chapter 2, the behaviour of the dogs turned out to be heterogeneous in several respects when we investigated it in more detail. The third indication for a strong genetic basis was the observation that aggression occurred more often in certain family groups than in others. This observation was made after grouping Golden Retrievers based on cluster analysis of their pedigrees (Knol *et al.* 1997). The formation of clusters with cluster analysis is rather arbitrary, so this study does not provide firm evidence for a strong genetic basis of Golden Retriever aggression.

In section 2.3 we described a more solid analysis of the heritability of Golden Retriever aggression. We obtained a heritability estimate of 0.77 for human-directed aggression. Although this heritability is high by all standards, we can draw only limited conclusions from these analyses because they were performed in a small group of dogs (n=316). Nevertheless, the results are in agreement with the hypothesis of a major gene involvement in Golden Retriever aggression. In the case of involvement of a major gene, we would expect to observe a more or less qualitative distribution of the phenotype because phenotypes of monogenic traits are usually qualitative (i.e. affected or unaffected) while polygenic traits usually display a continuous distribution (Bourdon 1997a). However, if there is a major gene in our families, the boundaries between the phenotypic categories may be blurred by other genetic variants with smaller effect and environmental influences. The more or less categorical owner impressions performed well in the quantitative analyses. This suggests that there is indeed a major aggression gene in our families of Golden Retrievers.

**Phenotype analysis**

We have used three methods for phenotyping: a behavioural test (section 2.1), a questionnaire for the dog owner (section 2.2), and a personal interview with the dog owner (sections 2.1 and 2.2). We obtained the most promising heritability estimates (i.e. high heritabilities with low standard errors) for the owner impressions collected during the personal interview.

A behavioural test is theoretically more objective than a questionnaire or personal interview. However, Jones and Gosling (2005) have reviewed studies of canine personality and noted that "*In theory, test batteries were the closest to achieving objectivity, but in practice the levels of objectivity actually attained varied substantially*." As we discussed in section 2.1, it is difficult to standardise behavioural tests. Our results demonstrate two additional drawbacks of using a behavioural test for phenotyping. As is explained in section 2.2, certain types of aggression could not be elicited in the test, e.g. territorial aggression. This problem could be solved in the future by performing tests in the home of the dog. However, home-testing would seriously compromise the standardisation of the test. The second drawback of a behavioural test is that the behaviour of a dog in a test may not be representative of its behaviour in everyday life. We observed that many dogs that were aggressive according to their owner showed little or no aggression in the test. This has been reported by others as well. For instance, Fuchs and colleagues (2005) noted that dog owners assigned higher scores for sharpness to their dogs than did judges in a behavioural test.

Sharpness was defined as the ability of the dog to react in an aggressive way towards a serious looking attack. Thus the validity of aggression measures collected with a behavioural test seems questionable. In other words, it is not clear what exactly is being measured with a test. Svartberg (2005) has suggested that aggression in the Swedish Dog Mentality Assessment test reflects aggression towards novel stimuli. This may also partially explain the discrepancy between the owner impression and test results in our work.

Owners are usually not considered to be skilled observers of dog behaviour (Galac and Knol 1997; Hart 1995; van der Borg *et al.* 1991). Nevertheless, our results suggest that owner-derived information was more suited for phenotyping than our behavioural test. In section 2.2 we concluded that the canine behavioural assessment and research questionnaire (CBARQ) is a promising tool for genetic studies because it is reliable and valid; and behavioural scores derived from it displayed sufficient variation in Golden Retriever families. However, using this questionnaire for phenotyping in our studies is complicated by two factors. First, most owners filled out the CBARQ several years after they had first reported aggression problems of their dog. Many dogs had become less aggressive in the course of time. As a result, CBARQ aggression scores were low for the majority of dogs. This may be an additional explanation for the poor performance of CBARQ items and CBARQ factors in our quantitative analyses in section 2.3. A possible solution for this problem is using the shortened CBARQ questions that referred to the behaviour of the dogs in the months prior to their first participation in the project. However, standard errors could not be estimated for the heritability estimations of these scores. It is therefore unclear whether they are suitable for genetic studies. Second, as we discussed in section 2.3, it is not straightforward which of the behavioural measures from the CBARQ should be used for phenotyping. The CBARQ items with high heritability estimates could be useful. However, each CBARQ item describes aggression under highly specific circumstances. There is probably not one specific genetic variation that influences variation in, for instance, "aggressiveness when the dog is approached by a strange adult approaching the leashed dog". One may miss important information when using such narrowly defined phenotypes, which also decreases statistical power. It may be preferable to use CBARQ scores or shortened CBARQ scores as phenotypes. However, these scores were based on phenotypic correlations. Phenotypic correlations are the result of both genetic and environmental factors. Consequently, CBARQ scores and shortened CBARQ scores may be a combination of genetically unrelated behaviours and using them as phenotypes may obscure genetic linkage or association.

We obtained the most promising heritability estimates (i.e. high heritabilities with low standard errors) for the owner impressions that we collected during a personal interview. These simple owner impressions outperformed the detailed behavioural measures that we obtained through the canine behavioural assessment and research questionnaire. Possible explanations for this surprising finding were already discussed in section 2.3. One additional explanation is that the owner impressions may be rather elementary phenotypes. In the personal interview, we asked the owners whether their dog was aggressive or not. If the owner considered the dog aggressive, we also asked whether the dog had actually ever bitten a person or another dog. This phenotype (scored as non-aggressive, threatens, or bites) may be very close to the genetic roots of the behaviour in the dogs that we studied.

In spite of the progress that we made with phenotyping, one major question remains unanswered: do the aggressive Golden Retrievers have a genetically based lowered threshold for aggressive behaviour in general or for a specific subclass of aggression? Our results are inconclusive with respect to this topic. We observed large phenotypic heterogeneity in the Golden Retrievers (section 2.1 and 2.2). This may be the result of both genetic and environmental heterogeneity. In section 2.3 we found that the estimated breeding values for human-directed aggression and dog-directed aggression were correlated, with a correlation coefficient of 0.4. We would expect this correlation to be higher if there is a major aggression gene influencing both traits. On the other hand, one of the two types of aggression may be influenced stronger by environmental influences than the other. We decided to be conservative in our first molecular analyses. We used scores of human-directed aggression for genotyping in section 3.4. Dogs that were exclusively aggressive to other dogs were not included in molecular analyses, in order to avoid possible misclassifications. Once we have actually found an aggression-causing mutation, it will be highly interesting to investigate its relationship with the various types of aggression.

The problem of phenotyping has received considerable attention in literature (e.g. Bearden *et al.* 2004). One of the solutions that have been proposed is the use of endophenotypes (Gottesman and Gould 2003). Endophenotypes, or intermediate phenotypes, are measurable components unseen by the unaided eye along the pathway between genotype and behaviour (Gottesman and Gould 2003). Endophenotypes may have a simpler genetic basis than the “end-phenotype”. One example of an endophenotype relevant to the study of aggressive behaviour is the level of neurotransmitter metabolites in cerebrospinal fluid (CSF) (Williams *et al.* 2003). Reisner and

colleagues (1996) have shown that these levels may correlate with aggressive behaviour in dogs. It would be interesting to include CSF levels of for instance 5-HIAA and HVA as phenotypes in a genetic study. Unfortunately it is not possible to implement this on a large scale. We were able to collect some CSF samples of aggressive Golden Retrievers before they were euthanatized. However, it turned out to be very difficult to obtain CSF samples from a proper control group. A control group for aggressive Golden Retrievers should consist of age- and sex-matched non-aggressive pet Golden Retrievers that do not suffer from a disease that affects CSF contents (Blennow *et al.* 1993; Higley *et al.* 1992; Kaplan *et al.* 1999; Reisner *et al.* 1996). Reisner *et al.* used laboratory animals as controls. This is suboptimal, because living conditions of dogs may affect CSF composition (Kraemer *et al.* 1989). In addition, phenotyping in pets is likely to be different from phenotyping in laboratory animals.

Other endophenotypes for aggression could be neuroreceptor binding indices measured with single-photon emission tomography (Peremans *et al.* 2003; 2005) or gene expression profiles in brain tissue. It would be highly interesting to compare brain gene expression patterns of aggressive and non-aggressive dogs. Behaviour is plastic, so we expect that many behavioural differences result from variation in gene expression rather than structural variation in genes (Hamer 2002; Saetre *et al.* 2004). Saetre and colleagues (2004) compared gene expression in the brain of dogs and wolves. They found altered expression of two neuropeptides, CALCB and NPY. They suggested that selection of dogs for behaviour during domestication has resulted in changes in mRNA expression patterns of several hypothalamic genes with multiple functions. Unfortunately, measuring gene expression in brain tissue is hampered by the same methodological difficulties as measuring CSF contents. It is more feasible to implement peripheral measures of neurotransmitter system activity in genetic studies of large numbers of dogs. Some (but not all) studies of human blood cell cultures have demonstrated a relationship between 5-HT functioning in these cells and aggressive behaviour (reviewed by Berman and Coccaro 1998). For instance, low platelet MAO discriminated violent offenders from non-violent offenders in a study of Belfrage *et al.* (1991). Male vervet monkeys with high rank within the social dominance hierarchy of a group demonstrate elevated levels of 5-HT in whole blood or in blood platelets (Raleigh *et al.* 1980; 1983).

## Environmental factors

The results in section 2.3 suggest that a large part of the variation in aggression in our Golden Retriever families can be attributed to genetic variation.

However, environmental factors undoubtedly influence the trait as well. Such environmental factors may include prenatal circumstances such as stress in the pregnant bitch (Serpell and Jagoe 1995) and early experiences such as maternal care (Scott and Fuller 1965; Serpell and Jagoe 1995), living conditions at the home of the breeder (Appleby *et al.* 2002), illness as a puppy (Podberscek and Serpell 1997b; Serpell and Jagoe 1995), and socialisation (Appleby *et al.* 2002; Houpt and Willis 2001; Scott and Fuller 1965). Scott and Fuller (1965) demonstrated that the experiences of puppies during the sensitive period (i.e. approximately 3-12 weeks of age) determine which animals and human beings will become their closest social relatives. Some of their experiments involved raising puppies with few human contacts. Such dogs showed extreme fearful behaviour and fear-motivated aggression towards humans later in life. Another potential environmental influence is the diet of the dog. The protein content of the diet and the amount of the amino acid tryptophan in the diet may influence the behaviour of a dog (DeNapoli *et al.* 2000; Dodman *et al.* 1996c; Houpt and Zicker 2003). Aggression can also be caused by pain and some diseases have specifically been reported to be associated with aggressive behaviour, e.g. epilepsy and hypothyroidism (see Reisner 1991 for a review).

There is a common belief that the major environmental cause of canine aggression is the relationship of the dog with its owner (Mugford 1995). We especially encountered this opinion in some dog breeders. However, scientific evidence for the influence of owner behaviour on canine aggression is sparse (O'Farrell 1997). Some scientists have found an association between anthropomorphic emotional involvement and dominance aggression in dogs (O'Farell 1995; 1997), but others failed to replicate this finding (Dodman *et al.* 1996b; Voith *et al.* 1992). Podsberscek and Serpell (1997a) studied the personality of owners of high- and low-aggressive English Cocker Spaniels using a questionnaire. Owners of high aggression dogs turned out to be more likely to be tense, emotionally less stable, shy and undisciplined than owners of low aggression dogs. Another study of Podberscek and Serpell (1997b) showed that high aggression Cocker Spaniels were slow in obeying commands, but there was no significant influence of feeding the dog before the owner eats, a lack of obedience training and playing competitive games with the dog. Jagoe and Serpell (1996) used retrospective data to investigate owner-dog interactions. They found that obedience training reduces the prevalence of competitive aggression. They also found a higher occurrence of territorial aggression in dogs that were always fed prior to the meal of their owners compared to dogs that were always fed after their owners had their meal. In addition, they found an association between sleeping close to the owner and competitive aggression. The latter finding was confirmed by Guy and

colleagues (2001). An association between first-time ownership and the prevalence of dominance-type aggression was reported by Jagoe and Serpell (1996). In conclusion, there seems to be a correlation between the behaviour of the owner and canine aggression. However, the observed correlations do not necessarily arise from a causal effect of the behaviour of the owner.

We observed a negative correlation between certain measures of obedience and aggression in the behavioural test (unpublished results). This correlation can be interpreted in several ways. One could argue that both poor obedience and aggression of the dog result from a lack of dominance of the owner. Conversely, one could argue that both arise from the aggressive or dominant nature of the dog. The nature versus nurture debate is outdated. Behavioural genetic researchers generally agree that behaviour is produced by intricate neural networks that are developed and maintained under influence of a variety of genes and environmental factors (Hamer 2002; Robinson 2004). Caspi and colleagues (2002) published an illustrative example of gene-environment interaction in antisocial behaviour. They studied a large cohort of male children from birth to adulthood to determine why some children who are maltreated grow up to develop antisocial behaviour, whereas others do not. They found a significant gene-environment interaction between a polymorphism of the gene encoding monoamine oxidase A ($MAO_A$) and maltreatment. Maltreated children with a genotype conferring high levels of $MAO_A$ expression were less likely to develop antisocial problems. Similarly, we expect that the phenotype of our Golden Retrievers is not only affected by the genetic predisposition for aggressiveness, but also by other factors such as dog-owner relationship.

## An integrative picture

In order to gain more insight in the factors that influence aggression, we have made a simplified and speculative diagram of information processing in the brain of a dog (Figure 1). Aggressive behaviour is normally triggered by a stimulus. This could be for instance a stranger approaching the home of the dog or the owner touching the head of the dog. The dog will first register the stimulus, i.e. it needs to see, hear, or smell the stimulus. The dog then interprets whether the stimulus poses a threat. In other words, the dog determines (probably unconsciously) whether the stimulus surpasses the norms of the dog. A stimulus can pose a threat to a dog in many ways. It could be a threat to the physical integrity of the dog, its owner, or its offspring (e.g. when a person attacks the dog, its owner, or its offspring). It could also be a threat to the social status of the dog, or to its food or reproductive chances. The third

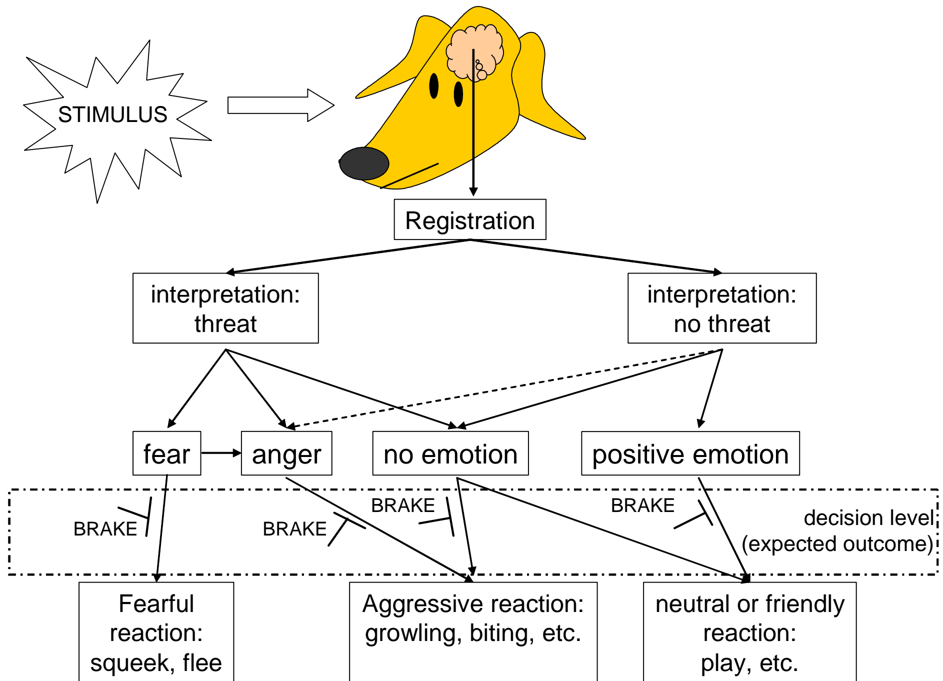



**Figure 1.** Simplified diagram of information processing in the brain of a dog. The process starts with a stimulus (e.g. a mailman approaching the home of the dog). The dog registers the stimulus and then evaluates (probably unconsciously) whether the stimulus poses a threat or not. If the stimulus is considered to be threatening, this may or may nor evoke fear in the dog. Fear will result in fearful behaviours like squeaking or trying to flee, or in aggressive behaviour like growling and biting. It is also possible that the stimulus does not evoke fear in the dog, but the dog reacts aggressively nevertheless. Theoretically, it is also possible that a dog reacts aggressively when it does not consider the stimulus as a threat. This is probably very rare, so the connection is presented as a dotted arrow. All these processes are executed by neural connections. Note that there is a "brake mechanism" in the neural pathways that lead to aggressive behaviour: there are inhibitory neurons in the brain that pose a threshold for aggression.

step is the reaction of the dog, which might be friendly, neutral, aggressive ("growling, biting, etc."), fearful ("squeaking, backing down, etc."), or a combination of these (e.g. fearful aggression). There is a decision level between the interpretation and the reaction of the dog.

Several pathways lead to aggressive behaviour in the scheme. The first pathway is mediated by the emotion of fear. Fear may either enhance or inhibit aggression, depending on the nature of the stimulus. For instance, the stimulus

is less likely to evoke aggression if the dog can escape from it. It is more likely to evoke aggression if the dog sees no opportunity to escape and if the dog thinks that the stimulus may be defeated. The likelihood of fear-motivated aggression thus depends on the expected outcome. Interpretation of the stimulus as a threat can also evoke an emotion that would be called anger in humans. This second pathway leads to fearless aggression. It is also possible that the stimulus does not elicit any emotion, but that the dog reacts aggressively nevertheless (i.e. a "cold", predatory type of aggression). The fourth pathway to aggression is rarely observed. Some dogs might behave aggressively in the absence of a threatening stimulus. This is truly abnormal behaviour, which may be comparable with psychopathy in humans. All pathways leading to aggression are equipped with a "brake". This brake is formed by inhibitory pathways that pose a threshold for aggression to occur.

Which factors influence this scheme of information processing? All steps, including the brakes, are executed by neural pathways. The strength of the neural connections determines the likelihood of a certain pathway to be activated. The strength of the connections is influenced by many factors, including genetic factors. For instance, polymorphism of regulatory DNA sequences results in individual variation in expression of neurotransmitter receptors in the brains of dogs. A difference in number of receptors may cause a difference in efficiency of synaptic transmission. In addition, there are polymorphisms in the coding sequence of genes encoding neurotransmitter receptors. Such polymorphisms may result in functional differences between the receptor proteins. Polymorphisms in genes that regulate the development of the nervous system can also lead to variation in the strength of neural connections. In addition to genetic factors, blood constituents such as androgens can influence neurotransmission. Such factors are regulated by both genetic and environmental influences (e.g. nutrition). The strength of neural connections is also strongly influenced by learning and early experience. It has been shown that early experiences influence the activity of various neurotransmitter systems (Kraemer 1986; Kraemer *et al.* 1989; Kraemer and Clarke 1990; Meyer and Bowman 1972; van den Berg *et al.* 1999). In conclusion, aggression is regulated by intricate neural networks that are influenced by a variety of genetic and environmental factors.

## Molecular genetic studies

We studied four serotonergic candidate genes and concluded that the genes are unlikely to play a major role in aggression in our Golden Retriever families. In addition to these four genes, we evaluated the canine tryptophan hydroxylase 2

gene (*tph2*) as a candidate. The enzyme tryptophan hydroxylase 2 catalyzes the rate-limiting step in the formation of serotonin in the brain (Walther *et al.* 2003; Zhang *et al.* 2004). The *TPH2* gene has been implicated in affective disorders in humans (Harvey *et al.* 2004; Zill *et al.* 2004). We genotyped three single nucleotide polymorphisms (SNPs) in introns of the canine *tph2* gene in eight discordant sibling pairs (i.e. an aggressive Golden Retriever with its non-aggressive sibling). The allele distribution of the SNPs in the Golden Retrievers did not indicate a role in aggressive behaviour (unpublished results of veterinary student Koen Elze).

The gene encoding monoamine oxidase A (*maoA*) is another potential candidate gene (Brunner *et al.* 1993a; 1993b; Cases *et al.* 1995; Caspi *et al.* 2002). Abeling *et al.* (1998) demonstrated that $MAO_A$ deficiency in human males can be diagnosed by measuring the ratio of substrates and metabolites of the $MAO_A$ enzyme in urine. We measured the concentrations of these substances in urine of six aggressive and six non-aggressive Golden Retrievers in a pilot study. The (unpublished) results did not point to a clear $MAO_A$ deficiency in the dogs. This suggests that aggression in the Golden Retrievers is not caused by a defect similar to the human Brunner syndrome. However, the results do not exclude *maoA* as a candidate because there may be a genetic variation in Golden Retrievers that causes a more subtle functional change rather than leading to complete $MAO_A$ deficiency.

Although we carefully selected the candidate genes, the candidate gene approach was similar to looking for a needle in a haystack. It is clear from the discussion in the previous paragraph that many genes may be involved in the aggressive phenotype. Aguirre-Hernández and Sargan (2005) have evaluated the candidate gene approach in the study of canine retinal diseases. They reported that only 3.4% of 377 published results of candidate gene studies identified the disease-causing mutation. Therefore, further progress in this project is expected with genome wide analyses. In genome-wide analyses there is no *a priori* assumption about which genes are involved in the phenotype. This opens the opportunity for finding genes that have not been associated with aggression to date. We are preparing genome-wide studies of aggression in Golden Retrievers. State-of-the-art canine genomic tools will be used to analyze 30,000 single nucleotide polymorphisms spread over the canine genome. If we succeed in mapping a genetic variant that is associated with aggression in Golden Retrievers, it will be very interesting to further study its relation to the variety of phenotypic measures that we obtained.

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# Summary

# Samenvatting

# Curriculum vitae

# List of publications

# Dankwoord

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# Summary

Aggression and Golden Retrievers? It might seem an unlikely combination since the majority of Golden Retrievers are friendly family pets. Nevertheless, we have encountered aggressive Golden Retrievers on a regular basis at the Clinic for Companion Animals at Utrecht University during the past few years. This aggressive behaviour might be strongly influenced by genes because it occurs more often in certain Golden Retriever families than in others. In this thesis I present our study of the genetic basis of aggression in Golden Retrievers. The final aim of this study was to identify genetic variations underlying the variation in aggressive behaviour in Golden Retrievers. The background of the research is described in **chapter 1.1**. We have collected DNA samples and behavioural information from 139 aggressive Goldens and 138 relatives since 1997.

In **chapter 1.2** I explain the importance of studies on the genetic basis of canine aggression. Such studies can answer several fundamental scientific questions. It is well-known that genes influence behaviour. For instance, differences in aggressiveness between human individuals are partially caused by the fact that different people have different variants of particular genes. However, it is difficult to determine how genes and environmental influences work together to produce behaviour. Dog breeds are very suitable for answering this question. A dog can only be registered as a member of a breed if both its sire and dam are registered members of the same breed. As a result, there is low genetic heterogeneity within dog breeds compared to human populations. Golden Retrievers are especially interesting for our study because they have been bred for friendliness.

Our research has an applied goal as well. Aggressive dogs can cause problems for their owner, victims, and for themselves. Aggression is a common reason for euthanatizing dogs. Behavioural therapy is the best treatment for aggression problems to date. Understanding the genetic factors underlying aggression will aid the development of additional methods of treatment. These can consist of pharmacological intervention for which scientific evidence is still sparse. In addition, aggression problems could be reduced through breeding programs.

**Chapter 1.3** contains a review of the literature on the genetics of canine aggression. The review shows that canine aggression is partly determined by heritable factors, but also that it is not yet known which genes are involved in the behaviour. However, there are indications that the

serotonergic neurotransmitter system plays a role in canine aggression. This system is involved in the communication between nerve cells in the brain.

A good method for measuring aggression is required to discover the genes that influence aggression. The development of such a method is the theme of **chapter 2**. We initially measured aggression with a behavioural (aggression) test (**chapter 2.1**); later we used a questionnaire for the dog owner (**chapter 2.2**). We expected the test to be an objective method for measuring aggression. The test consisted of 22 subtests during which the dog was provoked in various ways, including for instance, a subtest during which the feeding bowl of the dog was pulled away while the dog was eating from it. The tests were recorded on videotape and later they were analysed. We scored aggressive and fearful behavioural elements. Examples of aggressive behavioural elements are direct staring, freezing, growling, showing the teeth, and attacking. Examples of fearful behavioural elements include trembling, shrinking back, licking the beak, breaking eye contact, and hunching. We have calculated three types of aggression scores. Firstly, we calculated the "snap/attack score", which was the number of times the dog snapped or attacked during the test. Secondly, we calculated a "total aggression score". This stands for the number of times a dog showed aggressive behavioural elements. In other words, the total aggression score included threatening behaviour in addition to snapping and attacking. Thirdly, we studied the relationship between aggressive and fearful behavioural elements with a principal factor analysis. In this analysis, behavioural elements were grouped into factors based on their correlations. We then calculated factor scores for each dog that reflect the contribution of the behaviour of that particular dog to the formation of the factors.

We also interviewed the owner when a dog was subjected to the aggression test. This enabled us to compare the behaviour of the dog in the test with the opinion of the owner. We compared test scores of dogs that were aggressive according to their owners with those of dogs that were not aggressive according to their owners. In general, "aggressive dogs" had higher aggression scores in the test than "non-aggressive dogs". However, many dogs that were aggressive according to their owner showed little or no aggression in the test. The test thus seems to be insensitive, which is an important disadvantage of the test.

We started working with a questionnaire for the dog owners because the aggression test seemed to be unsuitable for phenotyping. In **chapter 2.2** we describe the results of this part of the research project. Our questionnaire was based on a questionnaire designed by James Serpell, an ethologist at the University of Pennsylvania. The questionnaire contained questions about fear,

anxiety, training, obedience, chasing, separation-related problems, excitability, and attention seeking in addition to the questions about aggression. Every question addressed the typical responses of the dog to specific situations. For instance: "Please indicate your dog's recent tendency to display aggressive behaviour when an unfamiliar person tried to pet the dog?". We included a brief explanation on the sorts of behavioural signs involved in the behaviour. Owners were asked to score the behaviour of their dog on a 5-point scale.

We used principal factor analysis to group the items of the questionnaire into factors. These included three aggression factors: stranger-directed aggression, owner-directed aggression, and dog-directed aggression. These factors were nearly identical to factors that were found by other researchers using the same questionnaire. This suggests that the factor solution is very reliable. We can use factor scores as a measure of aggressiveness. The aggression scores showed considerable variation in our dog families. This is a prerequisite for genetic studies.

We reanalysed the aggression test data using the knowledge that we obtained with the questionnaires. For instance, we performed a principal factor analysis of aggression scores during specific subtests. This resulted in three factors, two of which were similar to aggression factors from the questionnaire. We compared questionnaire scores and aggression test scores and concluded that the behaviour of the dogs in the test may not be representative for their behaviour in everyday life. For instance, we observed that many dogs were either fearful during the complete test, or that they showed little fear during the complete test. Therefore, it seems that fear during the test is not object-specific and it reflects the extent to which the dog is impressed by the test situation. The same may hold for aggressive behaviour in the test. We also observed that it was difficult to elicit owner-directed aggression in the test. We thus conclude that the questionnaires were more suitable for obtaining a valid impression of the behaviour of the dogs compared to the aggression test.

We collected a large number of measures of aggressiveness with the questionnaires and the owner interviews. The next question directed dealt with which of these measures show sufficient genetic variation to be useful for molecular genetic studies. In **chapter 2.3** we describe how we tried to answer this question with quantitative genetic research. We estimated heritabilities of several variables from the questionnaire and the interviews. Heritability is a measure of how strong genes influence a trait. A trait with a high heritability is the most suitable for molecular genetic studies. In order to estimate heritabilities, scores of dogs that are closely related (for example parents and their offspring) are compared to scores of dogs that are more distantly related. We obtained the most reliable heritability estimates for the simple owner

opinions we collected during the personal interviews. In addition, several aggression scores from the questionnaires showed clear genetic differences between the dogs.

The heritability estimations were very high (around 80%). This suggests that genes determine a large part of the variation in aggressiveness in the studied Golden Retrievers. It also suggests that a large part of the variation in aggressiveness is determined by variation in a small number of genes that each have a large effect on the behaviour. The results of the quantitative genetic analysis suggest that human-directed aggression and dog-directed aggression have a partially different genetic background. The results should be treated with caution because the number of dogs was very small. Also, the results cannot be extrapolated to other dog populations.

The final aim of our research was to identify mutations in genes that influence aggression in Golden Retrievers. Some knowledge of genetics is needed to be able to understand the principles of molecular genetic research. The body of a dog consists of billions of cells. Each cell has its own function. For example, a muscle cell should contract and a brain cell should transfer electric signals. The cells need a "computer code" to be able to perform their jobs. This code is written in the DNA. DNA is a very long molecule consisting of four components, the nucleotides A, C, G and T. Long strands of DNA are called chromosomes. Genes are small pieces of DNA that are found on chromosomes. The DNA of a dog contains about 30,000 genes. The DNA in the spaces between the genes can switch the genes on and off. In addition, there is a lot of DNA without a function or from which we do not yet understand the function.

Most body cells divide themselves on a regular basis. This enables the body to grow and maintain the tissues. Cell division also occurs during the formation of sperm and egg cells. During cell division, the chromosomes need to be copied. Mistakes are easily made during this process. If such a mistake is not corrected, a mutation arises. An example of a mutation is a point mutation, where one nucleotide (for instance A) is replaced by another (for instance C). A mutation can alter the function of a gene. If the mutation is located in one of the body cells, it usually does not have a large effect. However, mutations can also arise during the production of sperm- and egg cells. In this case, the descendants of the dog can inherit the mutated gene. All body cells of the descendant will contain the mutation. The mutation can give rise to a disease or it can influence the behaviour of the animal. Our hypothesis is that a mutation occurred in one of the genes that are involved in signal transduction in the brain in an ancestor of the aggressive Golden Retrievers. If dogs inherit this mutation from their parents, they have a predisposition to develop

aggressive behaviour. It is dependent on environmental influence whether the dog will indeed become aggressive and to what extent (for instance the quality of socialization and the way the owner treats the dog).

We have searched for mutations in a number of genes in the DNA of the Golden Retrievers. The genes we selected are involved in the serotonergic system. As mentioned before, this is one of the most important neurotransmitter systems in the brain. A number of scientific studies suggest that serotonin is involved in aggression in dogs. We have studied three genes that encode serotonin receptors (*htr1A*, *htr1B* and *htr2A*) and the gene encoding the serotonin transporter (*slc6A4*). The serotonin receptors enable serotonin to transfer its signal between nerve cells. In addition, the receptors regulate the activity of the nerve cells. The serotonin transporter is responsible for reuptake of serotonin from the clefts between nerve cells. The transporter is influenced by antidepressants such as Prozac. Studies in other animals and humans have shown that these genes play a role in aggression and anxiety. In **chapter 3.1, 3.2, and 3.3** we describe the isolation and characterization of these genes in the dog.

In **chapter 3.4** we compare the DNA sequence of the candidate genes between aggressive and non-aggressive dogs. The classification of “aggressive” and “non-aggressive” was based on the owner opinion in the personal interview because our quantitative genetic analysis showed that the owner opinion is very suitable for genetic analysis. We found no systematic differences between the DNA sequence of aggressive and non-aggressive dogs.

It is also possible that mutations in regulatory regions of the candidate genes influence aggression. The regulatory regions of many canine genes are not yet well defined. It is therefore time-consuming to search for mutations in these regions. However, it is possible to search for mutations indirectly using linkage analysis. The majority of regulatory regions are located close to the genes on the chromosome. Linkage analysis uses the linkage of marker alleles with mutations that are located nearby on the chromosome. Markers are pieces of DNA of which we know the location on the chromosome. A marker can for example be a gene or a short stretch of repetitive DNA sequence. If the marker displays small differences between dogs (or humans) it is referred to as a polymorphic marker. The various versions of markers are referred to as alleles. Markers have developed during evolution through mutations. They are spread over all chromosomes and can also be found in the chromosomal regions flanking our candidate genes. If a mutation arises in a regulatory region of a gene, that mutation will be flanked by specific alleles of the markers. If the puppies of a dog inherit the mutation, they will simultaneously inherit those specific marker alleles. If indeed the mutation influences aggressiveness, we

expect to see a disproportionably high frequency of certain marker alleles in aggressive dogs.

We thus investigated whether the aggressive dogs shared alleles of markers that are located close to or in the genes. We did not find such allele sharing. This suggests that the candidate genes are not likely to play a major role in aggression in the Golden Retrievers. As we discuss in the general discussion in **chapter 4**, we have also briefly investigated a fifth gene. This gene encodes an enzyme that is involved in the formation of serotonin. We found no evidence for involvement of this gene in aggression. Future research will reveal which genes do play a major role. We are currently working on a large-scale experiment in which the complete genetic material of aggressive and non-aggressive dogs is compared. If we succeed in identifying the mutations that play an important role in aggression, it will be very interesting to investigate their relation to the variety of aggression measures that we obtained.

# Samenvatting

Agressie en Golden Retrievers? Het lijkt een onwaarschijnlijke combinatie, want verreweg de meeste Golden Retrievers zijn een vriendelijke gezinshond. Toch zagen wij de afgelopen jaren op de Universiteitskliniek voor Gezelschapsdieren in Utrecht regelmatig agressieve Golden Retrievers. Dit agressieve gedrag wordt mogelijk sterk beïnvloed door genen, omdat agressie vaker voorkomt in bepaalde Golden Retriever families dan in andere. In dit proefschrift beschrijf ik ons onderzoek naar de genetische basis van agressie bij Golden Retrievers. Het einddoel van dit onderzoek is om mutaties op te sporen in genen die agressie bij Golden Retrievers beïnvloeden. De achtergrond van het onderzoek staat beschreven in **hoofdstuk 1.1**. Wij hebben sinds 1997 DNA-monsters en informatie over het gedrag verzameld van 139 agressieve Goldens en 138 aanverwanten.

In **hoofdstuk 1.2** leg ik uit waarom onderzoek naar de genetische basis van hondenagressie belangrijk is. Ten eerste dient het onderzoek om antwoord te krijgen op enkele fundamenteel wetenschappelijke vragen. Het is bekend dat genen invloed hebben op gedrag. Zo kan het feit dat niet elke mens even agressief is gedeeltelijk verklaard worden doordat verschillende mensen verschillende varianten van bepaalde genen hebben. Het is echter moeilijk om vast te stellen hoe genen en omgevingsinvloeden (zoals opvoeding) samen gedrag bepalen. Hondenrassen zijn heel geschikt om deze vraag te beantwoorden. Een hond krijgt namelijk alleen een stamboom als zijn beide ouders een stamboom hebben van hetzelfde ras. Hierdoor bevat een hondenras weinig genetische variatie vergeleken met een groep mensen. Dit betekent dat de verschillen in agressiviteit tussen honden worden veroorzaakt door variatie in een relatief klein aantal genen. Het opsporen van zulke genen is dus veel makkelijker bij honden dan bij mensen. Golden Retrievers zijn extra interessant voor het onderzoek omdat ze juist gefokt worden op vriendelijkheid.

Ons onderzoek dient ook een praktisch doel. Agressieve honden kunnen problemen veroorzaken voor hun eigenaar en eventuele slachtoffers, maar ook voor zichzelf. Het komt regelmatig voor dat eigenaren uiteindelijk beslissen om hun hond in te laten slapen vanwege zijn of haar agressieve gedrag. Op dit moment is gedragstherapie de beste mogelijkheid om gedragsproblemen te verhelpen. Pas wanneer we meer begrijpen van de oorzaken van agressief gedrag, kunnen we proberen aanvullende methodes te ontwikkelen voor het bestrijden van de problemen. Er zouden bijvoorbeeld medicijnen

ontwikkeld kunnen worden ter ondersteuning van gedragstherapie of een DNA test die door fokkers gebruikt kan worden om de goede kruising te bepalen.

In **Hoofdstuk 1.3** wordt een literatuuroverzicht gegeven van de genetica van agressie bij honden. Hieruit blijkt dat agressie bij honden gedeeltelijk erfelijk is, maar dat het nog niet precies bekend is welke genen betrokken zijn bij agressie. Er zijn aanwijzingen dat het serotonine-neurotransmittersysteem, dat betrokken is bij de prikkeloverdracht in de hersenen, een rol speelt bij hondenagressie.

We hebben een goede methode nodig om agressie te meten om te ontdekken welke genen agressie beïnvloeden. De ontwikkeling van zo een methode is het onderwerp van **hoofdstuk 2**. In eerste instantie hebben wij agressie gemeten met een agressie test (**hoofdstuk 2.1**); later met een vragenlijst voor de eigenaar (**hoofdstuk 2.2**). Wij verwachtten dat de test een objectieve methode zou zijn om agressie te meten. De test bestond uit 22 subtests waarin de hond op verschillende manieren uitgedaagd werd, bijvoorbeeld een subtest waarin de voerbak van de hond weggetrokken werd terwijl hij aan het eten was. De testen werden op video opgenomen, zodat we ze later konden analyseren. Tijdens het analyseren hebben we het optreden van agressieve en angstige gedragselementen geturfd. Voorbeelden van agressieve gedragselementen zijn aanstaren, verstarren, grommen, tanden laten zien en uitvallen. Voorbeelden van angstige gedragselementen zijn trillen, deinzen, bek aflikken, wegkijken en ineenduiken. Vervolgens hebben we drie typen agressiescores berekend. Ten eerste de "bijt/uitval score"; dit was het aantal keer dat de hond beet of uitviel tijdens de test. Ten tweede de "totale agressie score"; dit was het aantal keer dat de hond agressieve gedragselementen liet zien. Bij de tweede score werd dus behalve bijten en uitvallen ook dreigen meegerekend. Ten derde hebben we gekeken naar het verband tussen de agressieve en angstige gedragelementen met een principale factor analyse. Met zo een analyse worden op grond van de samenhang (correlaties) tussen de verschillende gedragelementen groepen van gedragselementen (factoren) gevormd. Voor elke hond kan dan een score op de factoren berekend worden, die zijn bijdrage aan zo een factor weergeeft.

Als een hond meedeed aan de agressietest, interviewden wij ook zijn of haar eigenaar. Hierdoor konden we het gedrag van de hond in de test vergelijken met het verhaal van de eigenaar. Voor een groep van 83 Golden Retrievers hebben wij de testscores vergeleken tussen de honden die volgens de eigenaar agressief waren en de honden die dat niet waren. Daaruit bleek dat "agressieve honden" over het algemeen hogere agressie scores in de test hadden dan "niet-agressieve honden". Er waren echter veel honden die geen of

weinig agressie in de test vertoonden, terwijl ze volgens hun eigenaar wel degelijk agressief waren. De agressie test is dus weinig gevoelig, wat een belangrijk nadeel van deze test is.

Omdat de test niet geschikt leek te zijn voor ons onderzoek, zijn wij gaan werken met vragenlijsten voor de eigenaar. In **hoofdstuk 2.2** worden de resultaten van dit deel van het onderzoek beschreven. Onze vragenlijst was gebaseerd op een vragenlijst van de Amerikaanse gedragsdeskundige James Serpell. De lijst bevatte niet alleen vragen over agressie, maar ook over angst, gehoorzaamheid, jachtgedrag, problemen bij alleen zijn, opwinding en aanhankelijkheid. In elke vraag werd een duidelijke situatie beschreven, bijvoorbeeld "In hoeverre had uw hond de afgelopen maanden de neiging om agressief gedrag te tonen als een onbekende persoon uw hond probeerde te aaien?" Er werd ook een korte beschrijving gegeven van de gedragskenmerken. We vroegen de eigenaar het gedrag van de hond weer te geven op een schaal van 0 tot 4.

Met een principale factor analyse hebben we de vragen uit de lijst gegroepeerd in factoren. Daarbij zaten drie agressiefactoren: agressie tegen vreemden, agressie tegen de eigenaar en agressie tegen honden. Deze factoren kwamen vrijwel exact overeen met factoren die in andere onderzoeken met dezelfde vragenlijst zijn gevonden. Dat wijst erop dat deze indeling in factoren heel betrouwbaar is. We kunnen scores op de factoren gebruiken als maat voor agressie. De scores op de factoren varieerden aanzienlijk binnen de Golden Retriever families. Dat is een belangrijke voorwaarde voor genetisch onderzoek.

Met de kennis uit de vragenlijsten in het achterhoofd, hebben we nieuwe analyses van de agressie testen gedaan. Zo hebben we een principale factor analyse gedaan op agressie scores tijdens de verschillende subtests. We vonden drie factoren, waarvan twee op agressie factoren uit de vragenlijst leken. Uit een vergelijking van scores uit de vragenlijsten en scores op de test factoren bleek dat het gedrag van de honden in de test wellicht niet representatief is voor hun gedrag in het dagelijkse leven. We zagen bijvoorbeeld dat honden meestal ofwel gedurende alle subtests heel bang waren, ofwel gedurende alle subtests weinig angst toonden. Het lijkt dus of de angst tijdens de test de mate weergeeft waarin de hond geïmponeerd is door de test situatie en niet zozeer een maat is voor angst voor specifieke stimuli. Dat zou ook kunnen gelden voor agressie in de test. We zagen ook dat "agressie tegen de eigenaar" moeilijk gemeten kan worden in de agressie test. We concluderen daarom dat we met vragenlijsten waarschijnlijk een beter beeld kunnen krijgen van het gedrag van de honden dan met de agressie test.

Met de vragenlijsten en de interviews van de eigenaren zijn er veel

verschillende maten van agressie van de honden verzameld. De volgende vraag was welke van deze maten sterk door genen beïnvloed worden. In **hoofdstuk 2.3** beschrijven we hoe we deze vraag proberen te beantwoorden met kwantitatief genetisch onderzoek. In dit onderzoek hebben wij de erfelijkheidsgraad geschat van diverse agressie scores uit de vragenlijst en het verhaal van de eigenaar. De erfelijkheidsgraad geeft aan in hoeverre genen bijdragen aan een kenmerk. Een kenmerk met een hoge erfelijkheidsgraad is het meest geschikt voor moleculair genetisch onderzoek. Om de erfelijkheidsgraad te berekenen, wordt gekeken in hoeverre scores van honden die nauw verwant zijn (bijvoorbeeld ouders en hun kinderen) meer op elkaar lijken dan scores van honden die weinig verwant aan elkaar zijn. Het bleek dat de meest betrouwbare schattingen van de erfelijkheidsgraad verkregen werden voor de agressieschatting door de eigenaar in het interview. Daarnaast gaven enkele scores op agressie naar mensen uit de vragenlijsten ook resultaten waarmee verder te werken is.

De schattingen van de erfelijkheidsgraad waren erg hoog (ongeveer 80%). Dat is een aanwijzing dat agressie in de Golden Retriever families die wij onderzocht hebben in belangrijke mate erfelijk bepaald is. Het suggereert ook dat de verschillen in agressiviteit tussen de honden in onze families bepaald worden door variatie in een klein aantal genen die elk een grote invloed hebben op het gedrag. Bovendien bleek uit dit onderzoek dat agressie tegen mensen en tegen honden weliswaar met elkaar correleren, maar dat het genetisch gezien waarschijnlijk twee aparte kenmerken zijn. Er moet wel voorzichtig omgegaan worden met conclusies uit dit kwantitatieve onderzoek omdat de onderzochte groep erg klein is. We kunnen de resultaten ook niet extrapoleren naar andere groepen honden.

Zoals al genoemd is, was het einddoel van ons onderzoek het opsporen van mutaties in genen die agressie bij Golden Retrievers beïnvloeden. Om te begrijpen hoe het moleculaire onderzoek precies werkt, is kennis van genetica nodig. Het lichaam van een hond bestaat uit triljoenen cellen. Elke cel heeft zijn eigen functie: een spiercel moet samen trekken, een hersencel moet elektrische signalen doorgeven, enzovoorts. Om hun functies naar behoren uit te kunnen voeren, hebben de cellen een "computercode" nodig. Die code staat geschreven in het DNA. DNA is een zeer lang molecuul, dat opgebouwd is uit vier verschillende bouwstenen, de nucleotiden A, C, G en T. Een lange sliert DNA wordt een chromosoom genoemd. Genen zijn stukjes DNA die verspreid liggen over de chromosomen. Het DNA van een hond bevat ongeveer 30000 genen. In de ruimtes tussen de genen ligt DNA dat de genen "aan en uit kan zetten". Daarnaast ligt er veel DNA wat geen functie heeft of waarvan we de functie nog niet begrijpen.

De meeste lichaamscellen delen zich regelmatig. Hierdoor kan het lichaam groeien en kunnen de weefsels in goede staat van onderhoud blijven. Bij de vorming van zaadcellen en eicellen vindt ook celdeling plaats. Tijdens een celdeling moeten de chromosomen gekopieerd worden. Hierbij worden gemakkelijk fouten gemaakt. Als zo een fout niet hersteld wordt, ontstaat een mutatie. Een voorbeeld hiervan is een puntmutatie, waarbij een nucleotide (bijvoorbeeld A) vervangen wordt door een andere (bijvoorbeeld C). Door een mutatie kan een gen een andere werking krijgen. Als de mutatie in een normale lichaamscel zit, heeft die veranderde genwerking meestal geen groot effect. Mutaties kunnen echter ook ontstaan tijdens de productie van zaad- of eicellen. In dat geval kan het gemuteerde gen aan nakomelingen worden doorgegeven en dan zullen alle lichaamscellen van de nakomeling de mutatie bevatten. De mutatie kan dan een ziekte veroorzaken of ervoor zorgen dat een dier zich anders gedraagt dan normaal. Wij veronderstellen dat er in een voorouder van de agressieve Golden Retrievers een mutatie is opgetreden in één van de genen die betrokken zijn bij prikkeloverdracht in de hersenen. Als honden deze mutatie van hun ouders erven, hebben ze een aanleg om agressief gedrag te ontwikkelen. Het hangt dan wel af van omgevingsinvloeden of de hond daadwerkelijk agressief wordt en ik welke mate (bijvoorbeeld de kwaliteit van de socialisatie van de hond en de manier waarop de baas met de hond omgaat).

Wij hebben in een aantal genen in het DNA van de Golden Retrievers gezocht naar mutaties. De genen die we geselecteerd hebben, zijn betrokken bij het serotonine systeem. Ik heb al eerder genoemd dat dit een van de belangrijkste neurotransmitter systemen in de hersenen is. Er zijn een aantal wetenschappelijke onderzoeken die suggereren dat serotonine betrokken is bij agressie bij honden. Wij hebben drie genen onderzocht die coderen voor serotonine receptoren (*htr1A*, *htr1B* en *htr2A*) en het gen dat codeert voor de serotonine transporter (*slc6A4*). De serotonine receptoren zorgen dat serotonine zijn signaal over kan brengen tussen verschillende zenuwcellen. Daarnaast regelen zij de activiteit van zenuwcellen. De serotonine transporter neemt serotonine op uit de ruimtes tussen zenuwcellen. De transporter wordt beïnvloed door bekende antidepressiva zoals Prozac. Uit onderzoek bij andere dieren en mensen is gebleken dat deze genen een rol spelen bij agressie en angst. In **hoofdstuk 3.1, 3.2 en 3.3** beschrijven we het kloneren en karakteriseren van deze genen bij de hond.

In **hoofdstuk 3.4** vergelijken we de DNA sequentie van de kandidaat-genen tussen agressieve en niet agressieve honden. De groepering in “agressief” en “niet agressief” hebben we gebaseerd op de agressieschatting door de eigenaar, omdat uit ons kwantitatief genetisch onderzoek bleek dat deze schatting heel geschikt was voor genetisch onderzoek. We vonden geen

systematische verschillen tussen de DNA sequentie van agressieve en niet-agressieve honden.

Het is echter ook mogelijk dat mutaties in regulerende gebieden van de kandidaat-genen de agressie veroorzaken. De meeste van zulke gebieden liggen vlakbij het gen op het chromosoom. Bij de hond zijn de regulerende gebieden van veel genen nog niet goed in kaart gebracht. Het is daarom lastig om in deze gebieden naar mutaties te zoeken. We kunnen echter wel indirect naar mutaties zoeken met zogenaamd koppelingsonderzoek. Bij koppelingsonderzoek maken we gebruik van de koppeling van allelen van merkers met mutaties die in hun buurt op het chromosoom liggen. Merkers zijn stukjes DNA die kleine verschillen vertonen tussen verschillende honden (of mensen). De verschillende varianten van merkers heten allelen. Eigenlijk is een gen ook een merker, want van de meeste genen bestaan meerdere allelen. Merkers zijn in de loop van de evolutie ontstaan door mutaties. Ze liggen verspreid over alle chromosomen, ook in de chromosomale gebieden rond onze kandidaat genen. Als er op een gegeven moment een mutatie ontstaat in een regulerend gebied van een gen, dan wordt die mutatie geflankeerd door specifieke allelen van merkers. Als de puppies van een hond de mutatie erven, erven ze tegelijk ook die specifieke merker-allelen. Als de mutatie invloed heeft op agressie, dan verwachten we een onevenredig hoge frequentie van een bepaald merker-allel bij agressieve honden.

In ons koppelingsonderzoek hebben we daarom gekeken of agressieve honden overeenkomsten hadden in de merkers die in of dicht bij de kandidaat-genen lagen. Een dergelijke overeenkomst vonden we niet. Dit betekent dat de vier genen waarschijnlijk geen belangrijke rol spelen bij agressie in de Golden Retrievers. Zoals besproken wordt in de algemene discussie in **hoofdstuk 4**, hebben we nog een vijfde gen kort onderzocht. Dit gen codeerde voor een enzym dat betrokken is bij de vorming van serotonine. Ook dit gen bleek waarschijnlijk geen belangrijke rol te spelen bij de agressie. Toekomstig onderzoek zal uit moeten wijzen welke genen er dan wel bij betrokken zijn. Wij werken momenteel aan een grootschalig experiment waarbij het complete genetische materiaal van agressieve en niet-agressieve honden vergeleken wordt. Als we erin slagen om een of meerdere mutaties aan te wijzen die een belangrijke rol spelen bij agressie, zal het heel interessant zijn om te onderzoeken wat het verband is tussen de mutaties en de verschillende soorten agressie die we gemeten hebben.

# Curriculum vitae and list of publications

Linda van den Berg was born on 1 August, 1977 in Oosterhout, the Netherlands. After graduating from the Mgr. Frencken College in Oosterhout, she started her study of biology at Utrecht University in the Netherlands in 1995. There, she specialized in fundamental biomedical sciences. During her first scientific internship, she conducted a psychophysical study on motion vision in cats, which was supervised by dr. M. Lankheet of the department of neuro-ethology at Utrecht University. The second scientific internship was performed at the department of neurology in the Haukeland Hospital in Bergen in Norway under supervision of prof. dr. C.A. Vedeler. Here, she studied Fc gamma receptor polymorphisms in DNA of Norwegian and Ethiopian people. Her interest in aggression research was already evident from her literature study where the relationship between aggression subtypes and autonomic arousal in humans was reviewed. This study was supervised by prof. dr. W. Matthys of the department of child and adolescent psychiatry at Utrecht University. Linda graduated *cum laude* in 2000. She increased her knowledge of the life sciences by studying veterinary medicine for two years. From September 2001 to April 2006 she worked on the PhD project described in this thesis.

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## Website

http://home.hccnet.nl/b.vanoost/

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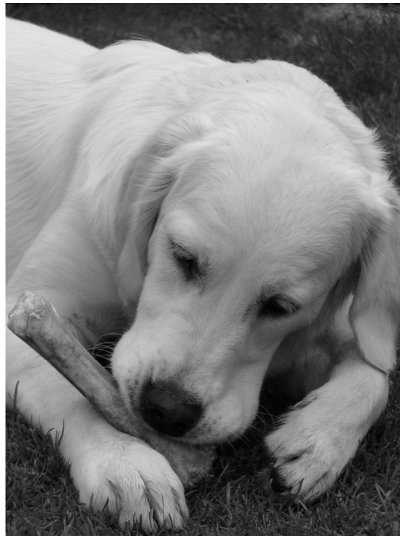

Anne; één van de deelnemers
(foto: Kim Boerkamp)