The Labrador Retriever as a Model of Naturally Occurring Myopia: Genetic and Environmental Contributions

Joanna Mary Black

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Optometry

The University of Auckland

2010

Abstract

Aims: To validate the dog as an animal model of naturally occurring myopia by identifying genetic and environmental factors which are associated with myopia development in the Labrador Retriever.

Methods: A large pedigree of Labrador Retrievers was phenotyped for refractive error. Using statistical (familial aggregation) analysis, refractive error data was used to investigate the inheritance of the trait within the pedigree. DNA samples were taken for later analysis. Potential environmental factors early in life were also studied in relation to adult refractive error; including birth weight, growth rate, season of birth, ocular pathology and litter size. DNA samples were extracted and a pilot genetic association study was designed to identify genetic loci for canine refractive error using a genome wide SNP array with a myopic case group and a non-myopic control group.

Results: A significant prevalence (37%) of myopia was found within the pedigree studied. Familial aggregation analysis demonstrated a significant genetic contribution, as well as showing a strong environmental contribution. Heritability of refractive error was 0.506. Smaller litters were shown to have significantly higher levels of myopia. No association was found between any environmental factors and adult refractive error, apart from season of birth in one age tertile. A genetic pilot study was designed to test for genetic loci which contribute to canine refractive error.

Conclusions: The Labrador Retriever is potentially an ideal animal model for the study of human myopia; the condition is naturally occurring in dogs, develops in a significant proportion of the population and can be phenotyped non-invasively. This study is the first to demonstrate the inheritance of myopia in any animal other than humans. The dog genome allows for myopia to be studied at a molecular level and due to the domesticated nature of the dog, breeding and environmental manipulations can also be conducted to identify further aspects of myopia causation.

Acknowledgements

First I would like to express my special thanks to my supervisor, Dr John Phillips, for all his time, support, and advice during my PhD. Also, special thanks to Andrew Collins for all his help, advice and support.

Thank you to Nicola Anstice for helping with so much refractive error testing. Thanks to Simon Back house for your help and invaluable support.

Thank you to Dr Sharon Browning for her extensive advice, guidance and help with statistics and familial aggregation analysis. Thank you also to Carol Chelimo, for her help with environmental statistical analysis

Thank you to Assoc Prof Andrew Shelling, Carey-Ann Eddy and Deborah Prendergast for your guidance and advice with the practical aspects of the genetics chapter.

Special thanks also to Nicola Cadogen, for all your help coordinating dogs for testing, and for supporting the project. Thanks also to Kim Malcolm, Lauren Elgie, Scott Bruce, Joe Walker and Helen McKenzie.

Thank you to both Professor Michael Kalloniatis and Professor Paul Donaldson for your guidance, and the support and resources of the department.

Lastly, thank you to my family and friends. To my parents, for your endless encouragement and support over the years. To Kevin, thank you for all your support, help, encouragement and patience; I couldn't have finished without you.

Table of Contents

List of Figures

List of Tables

Abbreviations

ACD	Anterior Chamber Depth
AD	Autosomal Dominant
AI	Artificial Insemination
AL	Axial Length
AR	Autosomal Recessive
AR	Auto-Refraction
BC	Border Collie
BMI	Body Mass Index
BW	Birth-Weight
CA	Corneal Astigmatism
CC	Corneal Curvature
CCR	Curly Coated Retriever
cM	CentiMorgan
cpd	Cycles Per Degree
D	Dioptre
DC	Dioptre - Cylindrical
DF	Degrees of Freedom
DS	Dioptre – Spherical
DZ	Dizygotic
F	Female
FDM	Form Deprivation Myopia
Gb	Gigabase
GDS	Guide Dog Services
GR	Golden Retriever
GS	German Shepherd
h^2	Heritability
IBD	Identical by Descent

IBS	Identical by State
K	Keratometry
LE	Left Eye
LIM	Lens induced myopia
LR	Labrador Retriever
M	Male
MZ	Monozygotic
OR	Odds Ratio
PERP	Pattern Evoked Retinal Potential
RE	Right Eye
Ret	Cycloplegic Retinoscopy
RNZFB	Royal New Zealand Foundation for the Blind
SER	Spherical Equivalent Refraction
SNP	Single Nucleotide Polymorphism
VECP	Visually Evoked Cortical Potential
WGS	Whole Genome Sequence
WGSA	Whole Genome Sampling Assay
µl	microlitre
µm	micrometre
λ	Recurrence risk ratio

Glossary

Allele	One of the alternative forms of a gene/genotype at a particular locus
Association Study	A statistical approach that tests for association between marker or candidate gene alleles and disease states
Candiae	The Family Canidae (canids, commonly known as either dogs or canines) includes wolves, dogs, foxes, coyotes, jackals, and so on. It is represented by 14 genera and approximately 34 species
centiMorgan	The unit of distance in genetic maps. Over short distances, if two genes are 1 cM apart then they will recombine on average 1% of the time
centromeric	The point at which a chromosome attaches to the spindle fibres during cell division.
Cephalic Index	Ratio (in percent) of the maximum breadth to the maximum length of a skull: Brachycephalic: short headed or broad headed, where the length of the cranium is shorter than the width, giving the top and sides of the cranium a round shape. Dolichocephalic: a long, rectangular head where the cranial length is greater than the cranial width. Mesocephalic: a square head where the ratio of cranial length is equal to cranial width, normal or medium proportion
Coefficient of Relationship	Denotes the proportion of genes that are held in common by two individuals as a result of direct or collateral relationship
Familial Aggregation	The occurrence of more cases of a given disorder in close relatives of a person with the disorder than in unrelated control families
Founder Effect	A high frequency of a particular allele in a population caused by it having been present in one or more members of a small number of individuals from whom the population is descended
Gigabase	One million DNA nucleotide bases
Genetic Drift	The random fluctuation in allele frequencies as genes are transmitted from one generation to the next
Haplotype	A set of closely linked alleles which are inherited together

Hardy Weinberg Equilibrium	A rule which relates the frequencies of genotypes at a locus in a population to the frequencies of the alleles at that locus.
Heritability	The extent to which a trait is genetically determined in a given population
Inbreeding Coefficient	inbreeding is computed as a percentage of the chance that two alleles are identical by descent. This percentage is called the "inbreeding coefficient"
Kinship Coefficient	The kinship coefficient is a measure of relatedness between two individuals. It represents the probability that two genes, sampled at random from each individual are identical
Laurasiatherian	Laurasiatheria is a clade of rank cohort or super-order, within the Placentalia (living) or Eutheria (Placentals and their extinct ancestors)
Linear Mixed Model	Linear mixed models (LMM) handle data where observations are not independent
Linkage	The tendency of genes close together on the same chromosome to be inherited together. It can be quantified and used as a mapping tool
Locus	A point on a chromosome or in a genome at which a specific gene or other marker is found. Often used incorrectly to mean gene
LOD Score	A statistic giving the level of confidence in an estimate of linkage distance between two loci
Microsatellite Marker	A DNA sequence of from 2 to 6 nucleotides which are tandemly repeated from 5 to 5,000 times (usual range 20 - 50 repeats). A locus containing a microsatellite is often polymorphic because of variation in the repeat number
Parity	In medicine, parity is a technical term that refers to the number of times a female animal has given birth
Population Bottleneck	A marked reduction in population size followed by the survival and expansion of a small random sample of the original population
Population Stratification	The division of a population into different ethnic groups with potentially different marker allele frequencies and different disease prevalence rates
Radiation Hybrid Map	Physical map of markers positioned on the basis of the frequency with which they are separated by radiation-induced breaks
Segregation Analysis	This technique is used to predict the probability that certain individuals will be of a certain genotype given information

	about the genotypes of ancestors and assumptions about the mode of inheritance. It can be used to distinguish between different models of inheritance
Telomere	The DNA structure which stabilises the ends of chromosomes
Variance components	A kind of hierarchical linear model. It assumes that the dataset being analysed consists of a hierarchy of different populations whose differences relate to that hierarchy
Whole genome shotgun	An approach to genomic sequencing that involves breaking the DNA into small pieces and cloning them into vectors, followed by sequencing the clones at random
Withers	The highest point on the back of an upright dog, on the ridge between its shoulder blades

Chapter 1. Literature Review and Aims

1.1. Background

1.1.1. Myopia

'Myopia is that form of refractive error wherein parallel rays of light come to a focus in front of the sentient layer of the retina when the eye is at rest' (Duke-Elder 1970).

Axial myopia results from an excessively long eye which causes light to focus in front of the retina and is usually due to an elongated vitreous chamber. Refractive myopia can also occur, a result of the refractive components of the eye being too powerful.

Refractive error is measured on a continuous scale from hyperopia through emmetropia to myopia. The dioptric level at which myopia is first defined is somewhat arbitrary, and has variable definitions in different studies. Myopia in most studies is defined as a refractive error of less than or equal to -0.50D in both primary meridians (Garner, Meng et al. 1990; Bullimore, Jones et al. 2002), although other studies have defined myopia as ≤-0.25D (Davitt, Dobson et al. 2005), or ≤-0.75D (Rosenfield and Gilmartin 1987; Zadnik, Mutti et al. 1993). Defining the level of myopia is important in studies of prevalence as it can greatly affect the results, particularly in age groups where myopia is just starting to develop.

Myopia can also be classified according to its degree. Hine (Hine 1949) originally classified myopia as low if less than 3D, as moderate if between -3D to -6D and high if more than -6D. An additional class was later added by Hirschberg (Rosenfield and Gilmartin 1998) of very high myopia, of greater than -15D.

1.1.2. Prevalence

Myopia is the most common ocular disorder in the developed world (Li, Guggenheim et al. 2009) and there is an enormous amount of literature available regarding the prevalence of myopia in a variety of countries. Only a brief review of some of these studies will be presented in this thesis as there are a number of comprehensive reviews on the subject (Fredrick 2002; Grosvenor 2003; Hyman 2007). Myopia is becoming increasingly prevalent

in the developed world, especially in East Asian countries where levels of myopia have reportedly reached 84% in some groups (Lin, Shih et al. 2004). The lowest levels reported to date have been in Nepal and Vanuatu at 1.2-2.9% (Grosvenor and Goss 1998; Garner, Owens et al. 1999; Garner, Stewart et al. 2004). The prevalence in New Zealand is unknown but the most recent Australian studies suggest that the level remains at approximately 8.4-14.7% (for ages between 4 and 12 years) which is similar to levels found in the early 1990s (Macfarlane 1987; Junghans and Crewther 2005). There is convincing evidence in a range of societies that levels of myopia are increasing compared to previous generations (Katz, Tielsch et al. 1997; Wu and Edwards 1999; Grosvenor 2003). There is also evidence that myopia has not increased in the last 30 years in other populations (Junghans and Crewther 2005).

1.1.3. Pathological Myopia

High myopia affects 27-33% of myopic people, comprising 1.7-2% in the general population (Sperduto, Seigel et al. 1983; Curtin 1985; Katz, Tielsch et al. 1997; Vongphanit, Mitchell et al. 2002). In some East Asian countries the prevalence of high myopia has been reported to be as high as 12-24% (Wong, Foster et al. 2000; Lin, Shih et al. 2004).

Myopia of any degree, particularly high myopia is associated with an increased risk of ocular pathology (Saw 2006). The reported risk of myopic retinopathy ranges from 0.3%-52.4% for levels of myopia ranging from -1.00D to $\leq$-9.00D (Vongphanit, Mitchell et al. 2002).

Pathological myopia shows various clinical signs including the presence of lacquer cracks, staphyloma, Fuchs' spot, myopic chorioretinal thinning/atrophy, tilting of the optic nerve and changes seen in the central retinal vessels (Vongphanit, Mitchell et al. 2002). There are also associated peripheral retinal degenerations associated with high myopia which increase the risks of retinal detachments such as lattice and pavingstone degenerations (Avila, Weiter et al. 1984). Certain clinical signs have been reported to have worse visual outcomes including patchy atrophy and choroidal neovascularisation compared to others such as lacquer cracks. Myopic patients also show an increased risk of cataract (McCarty, Mukesh et al. 1999), particularly posterior sub-capsular with myopia development before the age of 20 (Lim, Mitchell et al. 1999). Highly myopic patients show an increased rate of nuclear, cortical and posterior sub-capsular cataract (Lim, Mitchell et al. 1999). Myopic patients also exhibit an increased risk of glaucoma (Wensor, McCarty et al. 1999).

The incidence of blindness and visual impairment caused by myopic related disease is thought to be greatly underestimated (Li, Guggenheim et al. 2009). One report of Chinese adults listed myopic macular degeneration as the second leading cause of blindness after cataract (Liang, Friedman et al. 2008). Myopic chorioretinal degeneration has been reported as the fourth most frequent cause of blindness (Li, Cui et al. 2008), and myopic degeneration has been reported as the fourth most common cause of visual impairment in the working age population in Scotland (Bamashmus, Matlhaga et al. 2004)

1.1.4. Socio-Economic Impact

As myopia becomes more prevalent around the world, the socio-economic impact also increases. Even high levels of myopia can be corrected with negative spherical lenses, with resultant visual acuity usually reaching 6/6 acuity. However, developing myopia in early adolescence; the most common time of onset, leads to a lifetime of costs associated with refractive error; be it glasses, contact lenses or refractive surgery (Rose, Smith et al. 2001). There is also a significant cost associated with progressing myopia due to more frequent replacement of refractive correction, the need for regular eye examinations and the costs associated with the increased likelihood of ocular pathology which is commonplace in high myopia (Lim, Gazzard et al. 2009). It is estimated that the annual cost of myopia in Singapore is $148 million dollars (US) (Lim, Gazzard et al. 2009).

There is also a significant level of uncorrected myopia in regions of the world without access to refractive correction (Salomao, Mitsuhiro et al. 2009; Wright, Keeffe et al. 2009). Most literature estimates levels of uncorrected refractive error (including myopia, hyperopia and astigmatism), rather than myopia in isolation. It is estimated 12 million children are visually impaired worldwide from uncorrected or under corrected refractive error, with 1.4 million children under the age of 15 blind (Pararajasegaram 1999; WHO May 2009). There is also a significant loss in productivity associated with uncorrected refractive error (Smith, Frick et al. 2009).

1.1.5. Previous Animal Models

Myopia does not normally develop in animals. Most studies of refractive error in non-human species report emmetropia with some having a low hyperopic refraction (Smith, Harwerth et al. 1987; Murphy, Bellhorn et al. 1990; Murphy, Zadnik et al. 1992). However, lower levels of myopia are known to occur in animals with small eyes, due to the ‘small eye’ artefact, an

error which occurs during retinoscopy due to the light being reflected from the vitreo-retinal interface rather than at the photoreceptor level. This error increases as the eye size reduces and can led to an overestimation of hyperopia in animals such as mice and rats (Glickstein and Millodot 1970). The small eye artefact has been further supported by studies utilising visually evoked potentials for the estimation of refractive error when compared to retinosocpy and autorefraction in tree shrews (Norton, Wu et al. 2003) and rats (Mutti, Ver Hoeve et al. 1997). Higher levels of hyperopia are consistently found with retinoscopy and autorefraction when compared to the peak VEP response. However, there is evidence that the level of this artefact has previously been overestimated (Mutti, Ver Hoeve et al. 1997). Myopia in the lower half of the visual field has been reported in some species which require a closer focal length when looking down for foraging and hunting but still require clear vision in the distance to scan for predators. This occurs in a range of animals including the horse (Duke-Elder 1958), chick and turtle (Henze, Schaeffel et al. 2004). This change in focus is achieved by a variety of anatomical variations including a 'ramp' retina, where the superior retina, which decodes the lower visual field shows a change in shape to alter the focal length to allow a closer viewing distance (Schaeffel, Hagel et al. 1994). However, myopia can be induced in animals using a variety of environmental manipulations as follows:

1.1.6. Form Deprivation Myopia

Raviola and Wiesel (Wiesel and Raviola 1977) first showed the effects of lid suture on animal eyes when they were investigating the effect of visual deprivation on the developing visual system of young monkeys. They found that following lid-suture, the vitreous chamber of the eye grew abnormally long resulting in myopia. This effect has been termed Form Deprivation Myopia (FDM because vision was deprived completely by lid-suture or by the use of occludders which precluded form vision). FDM occurred in monkeys reared in the light but was not replicated in those reared in the dark (Raviola and Wiesel 1985) suggesting that the myopia developed due to disrupted visual input rather than to light attenuation. FDM has been demonstrated to occur in a range of species including tree shrews (Sherman, Norton et al. 1977), chicks (Wallman, Turkel et al. 1978), primates (Troilo and Judge 1993; Smith and Hung 1999), mice (Schaeffel, Burkhardt et al. 2004), fish (Shen, Vijayan et al. 2005) and guinea pigs (Howlett and McFadden 2006). Interestingly when the diffuser/sutures are removed vitreous chamber growth ceases, whilst anterior chamber growth continues until the

refractive state returns to emmetropia. This demonstrates the basis of emmetropisation as a visually guided process which is discussed later in this chapter.

1.1.7. Lens Induced Myopia

Refractive errors can also be induced in animals by the use of high powered negative or positive lenses. Application of lenses moves the focus either behind (negative lenses) or in front of the retina (positive lenses). Irving et al (Irving, Sivak et al. 1992) demonstrated that following a week of either positive or negative lens wear in chicks, the resultant refractive error was approximately equal to the power of the inducing lens up to the range of -10D to +15D. However, higher lens powers did not demonstrate the same relationship. Moreover, the effect appeared to be age dependent, with a more pronounced effect in younger chicks. Mammalian animal models including tree shrews (Shaikh, Siegwart et al. 1999; Siegwart and Norton 1999) and marmosets (Graham and Judge 1999) have also been shown to compensate for positive and negative lens wear in the range of +8D to -8D (although there was a less pronounced effect with positive lenses). Tree shrews have demonstrated more constant responses to positive lens defocus in the range of +4D to +6D (Metlapally and McBrien 2008). Studies with monkeys have been more variable, with some hyperopic refractions resulting from negative lens wear (Smith, Hung et al. 1994; Hung, Crawford et al. 1995) demonstrating a reduced range of lens compensation compared to chicks and other mammalian models (Irving, Sivak et al. 1992). However, if lens power is increased gradually in monkeys, the range becomes more comparable.

The relative strengths of myopic and hyperopic defocus have been studied by allowing experimental animals periods of unrestricted vision in combination with periods of defocus to study the potency of these signals. Myopic defocus seems to have a more potent effect on eye growth signals with significant levels of hyperopic refractive error developing with only an hour of myopic defocus out of a 12 hour light period in chicks (Schmid and Wildsoet 1996). The response to hyperopic defocus is not as established with as little as 3 hours of unrestricted vision preventing any compensatory eye growth. Similar results were found in tree shrew experiments (Shaikh, Siegwart et al. 1999), with minimal times of unrestricted vision preventing lens compensation during extended periods of hyperopic defocus. These findings suggest that myopic defocus provides a stronger stimulus for eye growth than hyperopic defocus.

The signal by which the eye is able to determine the direction of defocus is unknown. It has been demonstrated that part of the initial response of the eye to imposed defocus occurs in the choroid which either thickens to push the retina forward or thins to pull the retina back (Wildsoet and Wallman 1995; Smith and Hung 2000). In chicks these changes are observed rapidly with differences in choroidal thickness measurable after only 10 minutes of lens wear making it unlikely that the direction of defocus is determined by trial and error (Zhu, Park et al. 2005).

All of the animal experiments of myopia to date have used either FD or lenses to induce myopia in order to look at the physiological and anatomical changes associated with myopia development. These studies have provided valuable insights into the mechanisms behind myopia development and the resultant axial elongation. However, because this myopia is an induced condition it may not display some important characteristics of naturally developing myopia (Zadnik and Mutti 1995). It should also be noted that myopia induced in animal models tends to be of a high degree, develop in a matter of weeks and often resolves following the removal of the occluder/lenses. Mammalian and avian models of myopia also appear to demonstrate a different sensitive period to refractive error development in comparison to children (Zadnik and Mutti 1995). Animal models including chicks, primates and tree shrews are most sensitive to induced refractive error straight after birth, which does not correlate to juvenile onset myopia in humans (Zadnik and Mutti 1995).

1.1.8. Emmetropisation – Proof of a visually guided process

Emmetropisation is the process by which the co-ordinated growth of ocular components following birth produces an emmetropic refractive state. Emmetropisation has been described as being either a 'passive' process where the normal growth of ocular components reduces the level of refractive error during development, or as an 'active' process driven by visual input which influences eye growth (Saunders, Woodhouse et al. 1995).

There is a large body of evidence which supports emmetropisation being an active process driven by visual input (Wallman and Winawer 2004). This is supported by human pathology early in life, such as cataract or corneal opacification leading to axial elongation and high levels of myopia (Meyer, Mueller et al. 1999).

Chicks have been one of the most commonly used animal models in myopia research because of their ready availability, ease of handling, rapid development, low cost and rapid

development of refractive error (Chew and Balakrishnan 1992). Studies in chicks provided some of the first evidence for a visually guided emmetropisation system, with chicks being form deprived for two weeks developing myopia, which then recovered and rapidly returned to emmetropia within two weeks (Wallman and Adams 1987). The return to emmetropia was shown to be almost entirely due to a change in the VCD length rather than to other changes in ocular components (Wallman and Adams 1987).

Tree shrews also display recovery from FDM (Siegwart and Norton 1998), but the same result is not found in primate models which remain myopic following removal of the FD (Troilo, Nickla et al. 2000), and sometimes continue to become more myopic suggesting that growth is more unidirectional in this species (Troilo, Nickla et al. 2000).

Tree shrews that had the induced myopia corrected with minus lenses displayed no recovery response, again suggesting that the visual input is the major determinant of the resulting eye growth and emmetropisation (McBrien, Gentle et al. 1999).

It was also shown in chicks that depriving only a region of the retina resulted in axial elongation just in that region, with the non-deprived retinal regions remaining close to emmetropic (Wallman, Gottlieb et al. 1987). This suggests a local growth signal within the eye rather than a signal created by higher cortical processing. These findings were confirmed by studies which sectioned the optic nerve in chicks and primates removing higher growth signals, with FDM still developing in both chicks (Troilo, Gottlieb et al. 1987) and primates (Raviola and Wiesel 1985). A further study which investigated primates with the visual cortices removed also displayed FDM (Raviola and Wiesel 1985). It was found in chicks that optic nerve sectioning and form deprivation myopia resulted in a hyperopic refraction following recovery rather than emmetropia which suggests that higher processes may have a role in stopping ocular growth following visual signals (Troilo and Wallman 1991).

The age of the animal is important in studies of FDM, as younger animals display a higher level of myopia in comparison to adolescent animals who still demonstrate FDM but to a lesser extent (Troilo, Nickla et al. 2000). Recent studies have also demonstrated the importance of peripheral vision in the emmetropisation process, with complete foveal ablation not preventing recovery from FDM in monkeys. This suggests that the local growth signals which control ocular elongation may emanate from the peripheral rather than central retina (Smith, Ramamirtham et al. 2007).

1.1.9. Emmetropisation in human infants

Human infants tend to be born with a moderately hypermetropic refraction following a Gaussian distribution which is centred around +2.00D (Troilo 1992; Saunders, Woodhouse et al. 1995). On average, refraction declines until emmetropia is reached around 3 years of age. A hypermetropic refraction is found in 95% of the population at birth and 60% are emmetropic by 3 years of age (Montes-Mico and Ferrer-Blasco 2000). Adult refractions show a non-Gaussian distribution with an average emmetropic or slightly hyperopic refraction, and a standard deviation of only one dioptre (Saunders 1995).

Of the varying components controlling refractive status; including corneal curvature, anterior chamber depth, lenticular power and axial length; changes in axial length appear to be the greatest contributor to the shift from hyperopia to emmetropia after birth (McBrien and Barnes 1984; Mutti, Mitchell et al. 2005). Corneal curvature, anterior chamber depth and lens power all show normal distributions, with axial length (and refraction) showing a skewed distribution towards myopia (McBrien and Barnes 1984). The greatest rate of ocular growth is seen between birth and 3 years of age, after which growth slows with adult axial length reached between 13 and 15 years of age (Saw, Tong et al. 2004).

Children that demonstrate a myopic refraction at birth tend to become highly myopic as adults (Gwiazda, Thorn et al. 1993). Initially babies with myopic refractions show a trend towards emmetropia during the first 3 years of life, however their refractions gradually shift towards myopia from six years of age (Saunders 1995). One of the most accurate predictors of childhood myopia development is a relatively myopic refraction at the time of school entry (Mutti and Zadnik 1995). The prevalence of myopia in infants between 12-48 months is low compared to other age groups at 3% (Montes-Mico and Ferrer-Blasco 2000; Mayer, Hansen et al. 2001) but by nine years of age has reached 25.7% (Montes-Mico and Ferrer-Blasco 2000)

1.2. The Dog as a model of Myopia

1.2.1. Ancestry of the Dog

Canidae is a family composed of approximately 35 species divided among 10 genera (Ostrander, Galibert et al. 2000). The family consists of a variety of medium sized carnivores which successfully live in a wide variety of environments around the world. The genus Canis is amongst the most well known of this family including domestic and wild dogs (including dogs, jackals, coyotes and wolves).

1.2.2. From Wolf to Domestic Dog

The dog (species *canis familiaris*) was the first species to be domesticated, occurring more than 15,000 years ago (Ostrander, Galibert et al. 2000). At this time no other animal or plant species had been domesticated, and canine remains have been found in various locations around the world suggesting that their domestication spread quickly and widely. During the time of canine domestication, humans were nomadic hunter-gatherers (Price 1995).

Despite only diverging from their wild progenitor; the gray wolf (*Canis lupus*) (Wayne, Nash et al. 1987; Vila, Savolainen et al. 1997; Vila, Maldonado et al. 1999); so recently, domestic dogs show a dramatic range of phenotypic and behavioural diversity (Wayne 1986). Nonetheless the dog and wolf genomes are very similar, with analysis of mitochondrial DNA showing a difference of only 0.2% (Wayne 1993). The grey wolf is one of the only progenitor species of a domesticated animal that is still living in the wild, and available for direct comparison of domesticated and wild species. Wolfs tend to travel widely so that unlike the dog they have not become isolated in different geographic species, and have a high rate of 'gene flow' between different packs in geographic locations across Eurasia and Northern America (Vila, Savolainen et al. 1997).

1.2.3. Canine Diversity

There are more than 300 distinct breeds within the species *canis familiaris*. Dogs represent a species with extremes of morphology (Langston, Mellersh et al. 1999); from the Chihuahua which is less than 15 centimetres high to the Irish Wolfhound which stands at more than a metre. Dogs also have an extensive weight range of 1-90 kg (McGreevy, Grassi et al. 2004). Dogs have been specifically bred for a range of activities including hunting, herding and pulling and for a range of climates and conditions resulting in a range of phenotypes. Even

with their widely different physical characteristics, different breeds of dogs are still able to inter-breed and produce fertile offspring even with distant relations such as wolves and dingos. The long history of selective dog breeding has produced closely related individuals within each breed, which over time has concentrated genetic mutation and led to a range of inherited disorders (Ostrander and Kruglyak 2000). These disorders become increasingly concentrated where closely related individuals are bred to retain certain physical traits. This is particularly true in some breeds in which all dogs have been bred from a small number of founding individuals (Ostrander, Galibert et al. 2000). The effects of selective breeding become particularly problematic when a particular sire parents many puppies in a breed, known as the 'popular sire' effect, as it skews the genetic pool in one direction and does not allow the contribution of genes from other unrelated males (Ostrander 2006). Autosomal recessive and complex traits are the most problematic in this respect because it is difficult to detect carriers until they are bred with other like individuals. Often these diseases do not appear until later in life when several litters have been born and reproduced. These conditions may cause blindness (rod-cone dystrophy, glaucoma)(Kukekova, Nelson et al. 2006; Chen, Alyahya et al. 2008), deafness (Cargill, Famula et al. 2004), heart disease (cardiomyopathy) (Oyama 2008), orthopaedic conditions such as hip dysplasia (Janutta, Hamann et al. 2006), autoimmune diseases such as rheumatoid arthritis and lupus (Carter, Barnes et al. 1999; Jackson, Olivry et al. 2004) and dermatological conditions such as eczema (Tarpataki, Bigler et al. 2008). Autosomal conditions account for up to 60% of inherited diseases in dogs when pedigrees are analysed (Mellersh, Langston et al. 1997). However, it should be noted that a lack of genetic diversity is not automatically detrimental to a breed. If there is a lack of disease-causing homozygous gene-pairs then the health of the breed may not have increased pathology prevalence as a consequence (Patterson, Haskins et al. 1988). Many canine diseases also occur in humans but have been difficult to study in a human genetic setting. The presence of many of these conditions in both dogs and humans was the original argument for the induction of the canine genome project, which is discussed later in this chapter (Ostrander and Kruglyak 2000).

Numerous canine genetic registries have now been established to monitor genotypes and phenotypes in a range of dog breeds for diseases such as elbow and hip dysplasia and eye diseases such as progressive retinal atrophy (Gorig 2000; Sargan 2004).

1.2.4. Labrador Retrievers

Labrador Retrievers originated in Newfoundland and were bred from setters, spaniels and retrievers. They were bred to aid with bringing in fishing nets and catching fish which escaped from lines, making them water loving. Labrador Retrievers are medium to large in build, with males and females ranging in height (males 56-57cm, females 55-56cm at withers (see glossary)) and weight (average male weight 29-36kg, average female weight 24-31kg) when fully grown. They are currently the most popular breed of dog registered in the United Kingdom, United States and Canada. This is mainly due to their easygoing temperament which is known for intelligence, lack of aggression and willingness to please (www.thekennelclub.org.uk). They have 3 official coat colours (black, yellow or chocolate although other non-recognised variants exist) with iris colour being either brown or hazel.

1.2.5. Anatomy of the Canine Eye

Anatomically the canine and human eye is very similar. The main gross anatomical differences between adult human and adult dog eyes (medium sized breeds) are that adult dogs have a deeper anterior chamber by 0.5 to 1.3mm (human=3.11mm, Labrador=4.27mm), a lens which is thicker by 4mm (human=4mm, Labrador=7.83mm), a vitreous chamber depth that is shorter by 6-7mm and an overall axial length which is shorter by 1-2mm (human= 23.69mm, Labrador=22.12mm) (Mutti, Zadnik et al. 1999; Gorig 2000; Lee, Klein et al. 2009). Because dogs are active during the day and the night, they have much larger corneas and pupil sizes than humans which allows enhanced vision in low light (Nagayasu, Hirayanagi et al. 2009). Eye size is generally smaller in dogs than humans with eyeball radius in dogs ranging from 9.56-11.57 mm across a range of large and small breeds (McGreevy, Grassi et al. 2004). Eyeball radius correlates with skull length and width (McGreevy, Grassi et al. 2004), but not with cephalic index (see glossary).

The dog possesses a ‘visual streak’ (Mowat, Petersen-Jones et al. 2008), which is an area of increased photoreceptor density. The visual streak is located superior-temporally to the optic nerve head and extends longest nasally. The position and shape of the visual streak varies between and within different breeds and is classified as either moderate or pronounced depending on morphology (Peichl 1992). It is thought that the temporal part of the visual streak enhances binocular vision and the nasal extension aides the canine in scanning the horizon (Mowat, Petersen-Jones et al. 2008). The visual streak allows the dog to have high acuity over a wide field of view. Canine retinae have an area of high ganglion cell density

within the visual streak, the area centralis, which is equivalent to the human foveal area (Mowat, Petersen-Jones et al. 2008). Unlike the human fovea, the area centralis consists of both rods and cones (primarily L/M cones), with no rod free zone (Mowat, Petersen-Jones et al. 2008). The retina of the dog contains approximately 150,000 ganglion cells with a 75% crossover at the optic chiasm suggesting good binocular vision (Pretterer, Bubna-Littitz et al. 2004). Factors which contribute to retinal function and the ability to resolve an image include the ratio of photoreceptors to ganglion cells. It is known that in the human fovea this ratio is 1:1 and in the cat it is 4:1 (Goodchild, Ghosh et al. 1996). The ratio of photoreceptor number to ganglion cell number in the canine eyes is not known but is thought to be similar to that of the cat, due to similarities in the size of ganglion cells of the cat and the dog (Peichl 1992). Retinal ganglion cell distribution has been shown to be highly correlated with nose length in dogs (McGreevy, Grassi et al. 2004). This may explain the enhanced ability of dogs with longer nose length to hunt by sight. Dogs also have fewer optic nerve fibres than the human. Humans have 1.4 million nerve fibres whereas the dog has approximately 167,000 and the cat (Donaghy 1980) has 116,000 to 165,000. It is believed that the dog's visual acuity is limited by the retina and not by higher visual pathways or neural processing (Odom, Bromberg et al. 1983). This was based on evidence that when vision is measured using both visually evoked cortical potentials (VECP) and pattern evoked retinal responses (PERR), there is no significant difference (Odom, Bromberg et al. 1983). PERR measurements have been shown to be dependent on retinal ganglion cell function (Maffei 1981), whilst VECP and behavioural measurements are dependent on a combination of factors involving pre- and post- retinal processing The canine eye possesses a tapetum lucidum which is a broad reflective coloured band found in the superior half of the fundus. The tapetum is a highly cellular layer that is between 9 and 20 layers thick at its centre (Lesiuk and Braekevelt 1983; Wen, Sturman et al. 1985). The tapetum is green in the centre and yellow in the periphery and is interspersed with yellow dots (Lesiuk and Braekevelt 1983). The tapetum lies beneath the photoreceptor layer and is thought to maximize vision in dim light by reflecting incident light back through the retina and increasing apparent brightness (Miller and Murphy 1995; Pretterer, Bubna-Littitz et al. 2004).

One of greatest anatomical variants observed in different dog breeds is skull shape and size. There are 3 characteristic skull shapes in dogs; (brachycephalic, mesaticephalic and dolichocephalic (Ostrander 2006) (see glossary)). Skull anatomy can influence eye size and shape. One way of describing differing skull parameters is cephalic index (see glossary).

Cephalic index has been shown to correlate positively with the orientation of eyelid aperture and the ratio of peak ganglion cell number in the visual streak in relation to the area centralis in dogs (McGreevy, Grassi et al. 2004). Skull size also makes certain breeds more susceptible to conditions such as nasal tumours and mycotic rhinitis (Hayes, Wilson et al. 1982; Reif, Bruns et al. 1998). Skull shapes have been becoming more extreme over the last 50-100 years in some breeds as it tends to increase the chance of positive outcomes at dog shows. These changes in skull shape have occurred with increasing veterinary intervention allowing caesarean section, as many of the head sizes have become too large to allow natural whelping (Feldman and Nelson 2004).

1.2.6. The Canine Visual System

Canine visual performance is of particular importance as dogs carry out a range of functions within our community which require particular visual capacities. These include the ability of a herding dog to detect small changes in hand or arm posture seen from a distance to interpret commands, or a retriever dog who can visually detect and mentally remember the position where birds fall (Pretterer, Bubna-Littitz et al. 2004). It is interesting that even though dogs play such roles in our society, less is known about their visual systems than other animals such as monkeys, birds and rodents.

The dog has evolved to be a crepuscular species (most active at dawn and dusk) and so the canine visual system has adapted to low light levels. Dogs descended from omnivorous (mostly meat eating) and cursorial or 'running' ancestors (Ostrander and Giniger 1999). Although humans also evolved from omnivores, these ancestors were mostly fruit eating, diurnal and were tree dwelling. These differences in evolutionary development explain many of the differences between the canine and human visual systems. Humans have high acuity, good depth perception and excellent colour vision whereas the dog has good vision in dim light (Mowat, Petersen-Jones et al. 2008), as the dog's retina is predominantly populated by rod photoreceptors. Cones make up 3% of photoreceptors within dog and wolf retinas (Peichl 1991) (in comparison to humans, where cones make up 5% of photoreceptors). In the dog cones only make up a small proportion of photoreceptors within the area centralis, compared to the human; where the foveal region is purely cones (Koch and Rubin 1972). The presence of cones in the canine retina suggest that dogs may have some colour vision although behavioural studies (Coile, Pollitz et al. 1989) have found ambiguous results. Studies investigating dog vision using visually evoked potentials (Odom, Bromberg et al.

1983) and behavioural methods (Coile, Pollitz et al. 1989) have concluded that dogs have dichromatic colour vision with two classes of cone pigments, with spectral peaks at 429nm and 555nm (similar spectral sensitivities have been found in a range of canid species; the dog, island grey fox, red fox and arctic fox) (Miller and Murphy 1995). Canine rhodopsin which has peak sensitivity between 506nm and 510nm has a slightly different spectral sensitivity in comparison to human rhodopsin (peak sensitivity of 500nm). Also, rhodopsin takes over an hour to regenerate in dogs (compared to 30 minutes in humans) after extended periods of bright light (Jacobs, Deegan et al. 1993).

Dogs have a reduced amplitude of accommodation of 2DS to 3DS (Hess 1898). There is no recent evidence to support this value, and accommodation has not been measured using behavioural techniques in dogs. However, studies investigating the amplitude of accommodation in cats and rabbits have found similar levels using similar experimental methods as that utilised by Hess and Heine, through stimulation of the sympathetic nerve (Morgan 1939; Marg 1954) . Morgan et al measured one dog and found accommodation of approximately 1.5D (Morgan 1939).

The dog's binocular visual field is narrower than that of humans, being of 30º to 60º, with the total visual field in dogs being equal to 240º (Walls 1963).

There have been a number of studies measuring various aspects of the dog's visual capacity including visual acuity and brightness discrimination (Odom, Bromberg et al. 1983; Pretterer, Bubna-Littitz et al. 2004). Visual acuity has previously was found to vary between 3.96 to 6.21 cycles per degree (cpd) using behavioural techniques in mixed breed, medium sized dogs (Neuhaus 1967). Using visually evoked cortical potentials (VECP), canine visual acuity was measured as 4.62 cpd (2 beagles) (Bromberg 1980). Odom et al (Odom, Bromberg et al. 1983) measured visual acuity in the dog using pattern-evoked retinal potentials (PERP) and VECP in Beagles (Odom, Bromberg et al. 1983). Visual acuity had an average threshold of 12.59 cpd using VECP and 11.61cpd using PERP.

The temporal resolution of cones within the dog was studied by Peichl (Peichl 1992) who found that temporal resolution in dogs (70-80 Hz) was higher than that of humans (50-60Hz). In comparison the critical fusion frequency of rods seems to be similar (about 20 Hz) in dogs and humans (Coile, Pollitz et al. 1989). Brightness discrimination has also been studied in a number of breeds. Dogs were found to have the ability to discriminate brightness differences with a calculated Weber fraction of between 0.22 (German Shepherds) and 0.27 (Belgian

Shepherds) (Pretterer, Bubna-Littitz et al. 2004), indicating that the brightness discrimination of the dog is about half that of the human (Weber fraction = 0.11 (Griebel and Schmid 1997)).

1.2.7. Canine Ocular Pathology

Dogs exhibit many of the same ocular diseases as humans including cataracts (Curtis 1989), glaucoma (Chen, Alyahya et al. 2008), and retinal diseases such as progressive retinal atrophy (Ray, Baldwin et al. 1995), which resembles retinitis pigmentosa. Canine ocular pathology will be further discussed in chapter four.

1.2.8. The Canine Genome

The Dog Genome Project was a joint venture between the Fred Hutchison Cancer Research Centre and Whitehead/MIT Centre for Genome Research (Ostrander, Lindblad-Toh et al.). The first high quality draft (6x sequence) of the canine gene sequence was completed in 2004 (Ostrander and Comstock 2004). The dog genome was only the third mammalian genome to be fully sequenced (the first of the Laurasiatherian clade (see glossary)) and was a natural choice for gene sequencing to allow genetic models of human disease analogs in the species. Comparative mammalian models which have also been sequenced include the mouse (Guenet 2005), rat (Lazar, Moreno et al. 2005), cow (Zimin, Delcher et al. 2009), cat (Murphy, Davis et al. 2007) and guinea pig (Cui, McGregor et al. 2008). The draft canine genome sequence was based on the genetic code of a female boxer using a whole genome shotgun sequence approach (WGS) (see glossary)(Venter, Adams et al. 2001). The assembled sequence covered approximately 99% of the 2.4 gigabase (Gb – see glossary) genome. Along with the sequence, a 2.1 million single nucleotide polymorphism (SNP) map was created combining markers from the boxer, 10 other dog breeds and 5 other canid species forming a dense marker set for disease gene mapping and phylogenetic analyses. The SNP map will be further discussed in chapter 3.

The canine genome consists of 38 pairs of acrocentric autosomes and 2 metacentric sex chromosomes (Langston, Mellersh et al. 1997). Originally only 21 of the largest chromosome pairs could be characterised by cytogenic techniques due to size and morphology (Reimann, Bartnitzke et al. 1996; Switonski, Reimann et al. 1996; Mellersh, Langston et al. 1997), with the remaining 17 autosome pairs being identified through molecular techniques. The largest dog autosome, chromosome 1 is equivalent in size to

human chromosomes 9-12 (medium sized human chromosomes) and all but four, are smaller than human chromosome 19 (the smallest human chromosome). Analysis of the dog genome identified 19,120 protein coding genes, fewer than the ~22,000 human genes currently identified (Ostrander 2006).

The total size of the sequenced dog genome is ~2.4Gb, making the dog genome approximately 500 Mb (18%) smaller than the human genome. In comparison the mouse genome is 150Mb or 6% larger than the dog genome. A comparison between the dog and human genomes shows a higher level of shared ancestral sequences when compared to the human and mouse (Waterston, Lindblad-Toh et al. 2002), even though humans and mice share a more recent common ancestry (Waterston, Lindblad-Toh et al. 2002). The rate of divergence observed between the human and dog genomes are also similar, with an average divergence of ~0.35 substitutions/site (Lindblad-Toh, Wade et al. 2005).

Regions of the human and dog genome which share a common ancestry are referred to as regions of conserved synteny and are identified through sequence similarity. Inter-species genomic comparisons demonstrate both similarities in sequence structure due to shared ancestral genes and in differences which have occurred since their evolutionary divergence. When maps of syntenic regions are compared between different species it is observed that the human and rodent maps each cover ~94% of the dog genome, compared to the chicken genome which only covers ~76% (Lindblad-Toh, Wade et al. 2005).

One of the early steps in identifying genetic diseases within a species, given genomic information is the creation of genetic maps. Genetic maps are created in a variety of ways and allow for different genetic study strategies. The basic premise of these maps is that they create a framework of known genetic sequences at certain locations across the genome which can then be used to co-localise significant signals found during disease mapping. These maps are defined by the type of marker e.g. microsatellite/SNP, or the type of process used to create the map e.g. radiation hybrid/linkage map.

The creation of genetic linkage maps of the canine genome simplifies the study of genetic disorders. To be useable a genetic linkage map requires informative markers spaced no more than 10cM (centiMorgan) apart. Assuming that the canine genome was similar in size to the human, 350 informative markers would be required at equal intervals to provide full coverage of the genome (Langston, Mellersh et al. 1999). The first linkage map of the canine genome was completed in 1997 by Mellersh et al (Mellersh, Langston et al. 1997) based on the

tetranucleotide repeat markers (microsatellite markers) discovered by Francisco et al (Francisco, Langston et al. 1996), with 150 microsatellite markers characterised. This map was then expanded to produce a second generation map in 1999 (Neff, Broman et al. 1999), and expanded the number of markers from 150 to 276.

The existing microsatellite marker panels were then multiplexed to decrease the cost and difficulty of running canine genome scans. Multiplexing allows simultaneous amplification of multiple markers that are identified during gel electrophoresis by unique fluorescent labels (Eggleston, Irion et al. 2002). A further set was then multiplexed comprising 155 markers into 48 multiplex sets (Cargill, Clark et al. 2002). A third generation multiplexed linkage map has also been developed including 507 markers (Sargan, Aguirre-Hernandez et al. 2007).

Linkage marker panels have been used extensively to discover genetic loci for a range of canine disease including rod cone dystrophy (Kukekova, Nelson et al. 2006) and collie eye anomaly (Lowe, Kukekova et al. 2003).

A first generation canine Radiation Hybrid map (see glossary) was developed using 126 canine-rodent hybrid cell lines, consisting of 400 markers (218 gene mapped markers and 182 microsattellites) assigned to 57 RH groups (Guyon, Lorentzen et al. 2003). This then led to an expanded canine RH map consisting of 600 markers. The later creation of a combined RH and linkage map, lead to greater coverage of markers and genes to each chromosome (Mellersh, Hitte et al. 2000). The genetic loci for canine cataract have been identified using radiation hybrid mapping (Hunter, Sidjanin et al. 2006).

These maps have also been used to locate genes for canine diseases including narcolepsy in Doberman Pinschers (carnac-1), to canine chromosome 12 (human chromosome 6p21). This gene is now being investigated in humans in association with the biochemistry of sleep (Ostrander, Galibert et al. 2000).

1.2.9. The study of human disease using a canine model

The close inbreeding which has occurred within dog species over the last 300 years has created more than 400 hereditary canine diseases (Starkey, Scase et al. 2005) including cancer, heart disease, blindness and deafness, and at least half of these conditions have human disease equivalents. The dog shows a higher level of human-like diseases than any other species (Ostrander and Kruglyak 2000), and has the most reported hereditary conditions of any animal species. Often canine diseases are also given the same name as their human

counterparts and are caused by the same molecular mechanism with similar clinical course. The majority of canine diseases for which there has been an identified point mutation within a single gene are at the same locus as the human disease equivalents (Starkey, Scase et al. 2005). Canine genetic diseases are also commonly breed-specific having originated from an early ancestor making the gene simpler to identify due to minimal genetic variation within the breed. Minimal genetic variation occurs due to the nature of dog breeding, where economic pressures are removed and dogs are selected in terms of physical characteristics and behaviours. Other domestic animals which are bred as food sources such as cattle and sheep are selected against if they show any type of disease, particularly hereditary conditions. Whereas, dogs tend to get excellent veterinary care if showing disease, have vaccinations to reduce the amount of infectious disease and tend to have a low level of disease associated with poor nutrition or toxicity. This has led to discovery of thousands of variants of canine genetic disorders (Ostrander and Comstock 2004).

There are fewer ethical implications in trying novel therapeutic regimes in dogs which have naturally occurring rather than induced disease. Dogs respond in a physiologically similar way to humans when treated with pharmacological interventions, making them a common model for novel drug testing (Parker and Ostrander 2005). Dogs are becoming particularly important in cancer research as they show similar mechanisms of tumour development and have telomeres which closely resemble that of humans (Ollier, Kennedy et al. 2001; Thomas, Duke et al. 2008). The shorter life span of the dog also means that human conditions can be studied over a shorter length of time but still are comparable in terms of clinical course. Dogs share a common environment with man so is exposed to similar environmental triggers such as exposure to second hand smoke and toxins. Because other factors such as alcohol consumption and diet can be tightly controlled, causative factors can be more easily discovered. Compared to other animal models, dogs have the advantages of being less expensive, more available, have less infectious disease (due to a high level of immunisation) and are easier to work with than primates. Compared to mice, dogs are large enough for continuous sampling of tissue such as blood and bone marrow, and can have continual intravenous infusions (Ostrander 2006). Dogs have already been shown to be a valuable animal model in the development of protocols for bone marrow transplantation and underlying principles of MHC matching (Ollier, Kennedy et al. 2001).

Investigating canine disease models for human disease has the advantage of known ancestral backgrounds, often going back to original founding breed members. Dogs have a relatively

short gestation of 60 days and produce multiple offspring allowing relatively fast generation turnover which is useful in studying hereditary conditions. Being able to take DNA samples from three generations or more at any given time also strengthens the statistical power of genetic studies, as well as reducing required subject numbers. Selective breeding also allows patterns of inheritance and disease segregation to be examined.

Genetically dogs also present isolated breed populations whose genetic makeup differs significantly due to minimal genetic drift. Human genetic studies of disease often investigate isolated populations in an attempt to map disease genes. This approach has been successfully used to map genes for achromatopsia (Winick, Blundell et al. 1999) and mental illness in humans (Neuhausen 1999). Studies involving isolated Amish and Ashkenazi Jewish communities have also investigated genetic loci in myopia research (Stambolian, Ciner et al. 2005). The genetic variation between dog breeds has been shown to be greater than that seen between human populations, with 27% and 5-10% variation observed respectively (Parker, Kim et al. 2004), making them even more powerful for genetic studies of breed specific disease.

1.2.10. A current model of human eye disease in dogs

Retinitis Pigmentosa is an example of an ocular disease which has naturally occurring disease states in dogs (PRCD) and humans (RP), and presents an example of the success of the dog as a model of genetic human ocular disease. Unlike human studies, the canine model allows opportunities to directly investigate cell biology and therapeutic interventions.

Retinitis Pigmentosa (RP) is a blinding eye disease which currently affects 1 in 4000 in the human population. Several inheritance patterns have been distinguished in people including autosomal dominant, autosomal recessive and X-linked. Linkage studies have identified a number of possible loci for RP (Bayes, Martinez-Mir et al. 1996; Bardien, Ramesar et al. 1997; Sharon, Bruns et al. 2000). A group of similar naturally occurring conditions; the Progressive Retinal Atrophies (PRAs) occur in dogs. Progressive rod-cone degeneration (PRCD), one of the conditions within the PRA's has been of particular interest due to the similarities observed with human RP. A number of genes have been identified that cause the PRAs. A genome wide scan identified possible loci for PRCD, finding a very statistically significant loci close to the centromeric end of canine chromosome 9 (CFA9). The HLOD score at this position was 12.27, with a recombination fraction of 0.040 at marker FH.2263 (Acland, Ray et al. 1998). This region had been previously described by Werner et al

(Werner, Raducha et al. 1997) as having conserved synteny to human chromosome 17q, meaning that these loci are homologous in canine prcd and human RP17. The discovery of the canine prcd locus was the first utilization of a linkage map in canine genetics (Acland, Ray et al. 1998).

Interestingly some of these loci correspond to areas that have not previously been implicated in human RP, offering further candidate sites. Gene therapy has shown partial visual recovery in a form of this condition (Stieger and Lorenz 2008).

1.3. Aims

1.3.1. Aim 1: To measure refractive error and collect DNA samples in a large number of related dogs in order to identify those which are myopic

Previous studies have reported that various dog breeds show a range of refractive errors, with a significant proportion of dogs being myopic. The dog is the only species which shows naturally occurring myopia (Kubai, Bentley et al. 2008). The first aim was to test a large pedigree of purebred dogs to get a more accurate estimate of refractive error across a range of generations and litters. This was a precursory step to statistically testing if refractive error within the pedigree was inherited. Myopia shows a strong genetic component in human families, but this had not demonstrated in a non-human species. Labrador Retrievers were chosen because they are the only breed of dog in which myopia has been shown to be axial in nature (Mutti, Zadnik et al. 1999).

1.3.2. Aim 2: To construct large pedigrees to identify the pattern of inheritance of myopia

The first step in any familial study of disease is assessing statistically if the disease occurs because of inheritance (genetic components) or whether it is due to the environment (environmental and spurious causation). Familial aggregation and heritability calculations are a way of detecting whether a trait is inherited or is more likely to have been caused by environmental factors. Familial aggregation involves studying the pattern of a trait within a family, without identifying a specific genetic model. Heritability is the variance of a phenotype due to genotype. Once Aim One was completed, the pattern of refractive error was analysed using variance component modelling over the entire Labrador Retriever pedigree. Due to the pedigree structure and high level of consanguinity a mode of transmission (ie. dominant/recessive) could not be tested.

Chapter Two of this thesis outlines the literature and background supporting aims one and two and then outlines the methods used and results obtained in the familial aggregation portion of the project. Chapter Two also describes the recruitment of subjects used throughout the project (aims 1-4), methods of refractive error measurement and the statistical analysis which supported the continuation of the project to address aims 3 and 4 (below). It should be noted that two groups of Labrador Retrievers were phenotyped in this project and

that chapter two discusses only the large pedigree used for familial aggregation analysis (Black, Browning et al. 2008).

1.3.3. Aim 3: To design a genetic association study with the potential to identify candidate regions for canine refractive error

Once Aim 2 established that refractive error in this family did have a significant genetic component, designing a pilot genotyping study was the appropriate next step in attempting to identify genetic loci for canine refractive error.

There are several approaches that could have been used to address this aim, but it was decided to use the recently developed canine SNP array, as this gives genome-wide coverage and is the latest available technology. The canine SNP array was recently developed by the Broad Institute (Karlsson, Baranowska et al. 2007). The sampling strategy was to design a case-control study with 50 myopic dogs from the pedigree constructed in Aim 1, and a control group of non-myopic, unrelated Labrador Retrievers from other breeding colonies.

Chapter Three describes the genetic study which was designed to investigate whether a region of interest could be identified that may correlate to the myopic phenotype in Labrador Retrievers. Chapter Three followed on from chapter two which had demonstrated that refractive error in this pedigree had a significant genetic component (as in humans). This part of the project designed a case-control association design i.e. a group of Labradors which was myopic (from the population used in Chapter Two – Familial Aggregation), and a separate non-myopic unrelated group of Labradors from breeders throughout New Zealand. This chapter includes the construction of relationship variables which are required prior to genotyping, and was particularly important due to the high level of inbreeding within our pedigree population.

1.3.4. Aim 4: To identify possible environmental factors which could contribute to refractive error in the Labrador Retriever

Following the findings of Aim 1, that (a) refractive error in the studied pedigree was inherited and (b) that smaller litters demonstrated higher levels of myopia, further investigation of environmental influences was warranted. Working with the same breeding colony identified for Aim 1, we gained access to breeding records which detailed litter size, date of birth, birthweight and information about post-natal health. These variables were analysed in

conjunction with adult refractive error to look for relationships between environmental factors.

Chapter Four investigates the environmental factors which may influence refractive error development in Labrador Retrievers. This chapter also followed on from chapter two because although the familial aggregation analysis showed a genetic component, there was also a large amount of variance which was due to non-genetic factors e.g. environment. This again studied the colony of dogs used in chapter two. These dogs all shared a common environment in the early stages of their development, which provided a unique opportunity for study. The major factors which were investigated both independently and in relation to adult refractive error were birth weight, growth rate, litter size, litter cohort and season of birth.

Chapter 2. Familial Aggregation Analysis

2.1. Introduction

2.1.1. Familial Aggregation Studies

The first step in analysing the inheritance of refractive error in Labraodor Retreivers is to statistically test a pedigree to determine if refractive errors exhibit a heritable component or are more likely to occur by chance, which has not been studied previously. However, many studies of human refraction in families have studied the pattern of refractive errors through small nuclear and larger multigenerational families. Chapter 2 will discuss the underlying theory behind the statistical techniques for studying disease heritability, the current application of these techniques in human studies of refractive error, and the current literature available concerning the level and development of refractive error in animals; particularly dogs.

The initial step to investigate the inheritance of any trait is to determine whether the phenotype segregates in families, before investigating a particular mode of inheritance. This is to differentiate genetic versus environmental contributions. When a disease phenotype is found to segregate in one family it is due to the sharing of alleles (see glossary) from shared ancestors. When an allele is the same in two related individuals because it is inherited from a shared ancestor, it is said to be identical by descent (IBD) (Fernando, Mota et al. 2005). When the same allele is found in two random individuals, and shows the same DNA composition and function but does not come from a shared ancestor it is said to be identical by state (IBS). Many tests involving inheritance of disease depend on IBD statistics. For example the expected proportion of alleles shared IBD between siblings is 0.5. This proportion can be measured by the coefficient of relationship and equals 2^{-2R}, where R is the degree of relationship, that is, 0 for identical twins, 1 for first degree relatives (siblings, parent-offspring), 2 for second degree relatives (grandparents, half-sibs) and 3 for third degree relatives such as first cousins.

Another similar measure of relationship is the kinship coefficient, defined as the probability that a randomly selected pair of alleles, one from each individual is IBD and is equal to half the coefficient of relationship (Elston 2000). The final measure of relationship is the Wright's coefficient of inbreeding, which is similar, but is measured for a single individual.

Wright's inbreeding coefficient is the probability that identical alleles inherited by an animal are the result of transmission from a common ancestor, through the sire and the dam (Wright 1922).

When testing disease phenotypes within families, intraclass correlations from analysis of variance give correlations from different types of familial relationships such as parent child or sibling relative pairs. Correlations between relation pairs are a more appropriate test than standard Pearson correlation coefficients as it more accurately orders relationships. Following the calculation of correlations for each type of relationship of a given disease state, these are then compared for predicted correlations in a model of genetic and environmental factors to give the best fitting model. The conceptual model for this analysis is a linear model of the form:

$Yij = \mu + G_{ij} + Cij + Eij$ with variance of Y equal to:

$$Var(Y) = \sigma^2 = \sigma^2_G + \sigma^2_C + \sigma^2_E$$

Where G, C and E represent unobservable random variables for genotype (G), shared environment (C) and independent environment (E), which are by definition independent.

2.1.2. Heritability

Heritability as used in common English is defined as '"the quality of being heritable, or capable of being inherited" (Stoltenberg 1997). The word heritability is often used interchangedly with heredity, inherited and heritable; terms which all imply the passing of a trait from parent to offspring. However these terms are often confused with the statistic 'heritability' (h^2) which leads to confusion about what heritability estimates imply. Heritability was first described by Fisher in 1918 as a way of resolving polygenic inheritance (Fisher 1918). Later, Fisher's fundamental theorem of natural selection implied that the speed of achieving desirable phenotypes through artificial selection is determined by the magnitude of h^2 (Fisher 1958).

The definition of 'heritability' as a statistic is 'the proportion of the total phenotypic variance that is associated with genetic variance in a specific sample with a specific genetic composition and environmental context' (Vitzthum 2003).

Broad sense heritability is defined as the proportion of variance due to genotype σ^2_G/ σ^2. Heritability in twin pairs can be calculated directly from intraclass correlations if it is assumed that environmental factors are shared.

The genetic variance can then be further divided into additive effects of individual alleles (additive genetic variance), same locus interaction between alleles σ^2_D (dominance variance) and σ^2_I, interaction at different loci (the epistatic variance). As described above; broad sense heritability is the proportion of total variance attributable to genetic (or non-environmental) variance (additive and dominant genetic variance), however as an individual only inherits one allele from each parent, the dominant component (due to pairs of homologous alleles) cannot be transmitted. Narrow sense heritability refers to just the additive component (which is transmissible and amenable to selection); σ^2_A/ σ^2. It is important to note that this does not necessarily imply one major gene, two additive genes or several genes of small effect; this is tested by segregation analysis.

It should also be noted that studies of heritability cannot be compared if population samples come from different environments. Heritability estimates are always specific to a particular sample. If the same sample was measured twice, each time with different environmental factors, the heritability estimates would also differ even though the genotypes would be unaltered. Heritability statistics cannot provide information about the mode of inheritance, number of loci, the location a locus, or the control of the phenotype by the loci. For example; if a study found a trait had a heritability value of 0.6, this does not mean that 60% of the cause of that trait in an individual is caused by their genotype, rather it means that in the sample studied genetic variance is 0.6, caused by non-environmental factors. As heritability is a ratio of additive genetic variance as a proportion of total phenotypic variance, values are between 0 and 1, but are often also expressed as a percentage.

2.1.3. Segregation Analysis

Segregation analysis is a statistical method of studying the disease patterns in pedigrees to ascertain the most likely mode of transmission; such as dominant, recessive, co-dominant or x-linked transmission (Elston 1981). Complex segregation analysis relies on the principles of Mendelian inheritance and Mendelian transmission, and single genes of major effect. Segregation analysis is commonly used prior to linkage analysis, particularly parametric or LOD based analysis (see glossary) where specific parameters are required.

2.1.4. Myopia Heritability – Twin studies

Many studies in a variety of populations have shown a strong role for genetics in determining ocular refraction (Teikari, Koskenvuo et al. 1990; Teikari, Kaprio et al. 1992; Angi, Clementi et al. 1993; Hammond, Snieder et al. 2001). Studies of twins, in which there is minimal genetic variability in comparison to regular siblings (100% conservation of genetic material in monozygotic twins (MZ), 50% conservation in dizygotic (DZ) twins) (Feldkamper and Schaeffel 2003), have shown the strongest evidence for genetically determined refractive status. One of the advantages of twin studies is that they allow modelling of both genetic and environmental influences (Dirani, Chamberlain et al. 2006), both in terms of comparing MZ/DZ twins that have been raised in the same environment and those that have been reared apart, as in adoption studies (Knobloch, Leavenworth et al. 1985). Twin studies also remove the difference of age from pairwise comparisons. By directly comparing the correlation of refractive error between MZ/DZ twins, heritability calculations can be made. There have been numerous small scale twin studies reported over the last century, mostly in the form of clinical observations or case studies, and these have been reviewed (Karlsson 1974), with the finding that overall MZ twin pairs showed a 95% concordance rate of refractive error compared to 29% for DZ pairs. However, one of the major difficulties with early studies was correctly identifying zygosity and excluding other causative pathology. Sorsby et al conducted the first large scale twin study which concluded that MZ pairs had a much higher concordance rate for myopia (>0.9) than DZ pairs (<0.25), as well as a high correlation rate of other ocular parameters (Sorsby and Fraser 1964).

Heritability of refractive error in more recent twin studies has shown variable results, but has continually demonstrated that MZ twin pairs show significantly higher h^2 values in comparison to DZ pairs implying genetic causation (Karlsson 1974; Hammond, Snieder et al. 2001; Lyhne, Sjolie et al. 2001; Dirani, Chamberlain et al. 2006; Lopes, Andrew et al. 2009). Reported heritability values vary between 0.11-0.98, with the majority falling between 0.72-0.98 (Sorsby and Fraser 1964; Lin and Chen 1987; Teikari, Kaprio et al. 1992; Angi, Clementi et al. 1993; Hammond, Snieder et al. 2001; Lyhne, Sjolie et al. 2001; Lopes, Andrew et al. 2009). More recent studies have used more sophisticated statistical models utilising multivariate modelling allowing more variables to be studied.

The heritability of high myopia (>-5.99D) has not been studied as thoroughly as low/moderate myopia, but has been shown to be more heritable in some studies (De Jong,

Oostra et al. 1993; Guggenheim, Kirov et al. 2000; Liang, Yen et al. 2004), while other studies have reported lower heritability in high myopia (Karlsson 1974; Lin and Chen 1987).

However, it should be noted that all of these twin studies have different criteria in terms of sample size, age of twin pairs, refractive error distribution and ethnic background. Some studies have also not measured refractive error under cycloplegia. In particular the study conducted by Angi et al (Angi, Clementi et al. 1993), does not take into account adult refractive error, particularly myopia which develops during adolescence as the twin pairs were between 3 and 7 years of age. Dirani et al went on to study the prevalence of adult onset myopia (defined as first optical correction at age 18 or older) in the GEM cohort of twin pairs, and showed that adult-onset myopia also shows a significant inherited component, with a significantly higher correlation in MZ pairs (r=0.61) compared to DZ pairs (r=0.16) (Dirani, Shekar et al. 2008).

The prevalence of refractive errors in twins has in most studies been shown to demonstrate no significant difference in comparison to singletons. This is an important finding as premature babies have been shown to have a higher prevalence of refractive error (Cosgrave, Scott et al. 2008), including myopia (O'Connor, Wilson et al. 2007)(independent of retinopathy of prematurity) (Quinn, Dobson et al. 1998), although this association has not been observed in all studies (Saw and Chew 1997). It also appears that low birth weight may be a greater predictor of myopia development than gestation (Varughese, Varghese et al. 2005). Twins have a significantly higher prevalence of premature birth (Bagchi and Salihu 2006). Some studies have demonstrated a difference in the prevalence rates of hyperopia and astigmatism between twins and singletons (Hur, Zheng et al. 2009), but not in myopia.

2.1.5. Heritability of Ocular Biometric Characteristics

Along with the genetic and environmental variance associated with refractive error, ocular parameters affecting refraction have also been investigated in twin pairs and in family studies; including corneal curvature, corneal astigmatism and corneal thickness, axial length, and anterior chamber depth. All of these parameters have been demonstrated to display significant genetic variance and hence they show greater correlations in monozygotic compared to dizygotic twins (Lyhne, Sjolie et al. 2001; Dirani, Chamberlain et al. 2006; He, Wang et al. 2008; Zheng, Ge et al. 2008). Many of these parameters show significantly different heritability values depending on gender (Mash, Hegmann et al. 1975; Biino, Palmas et al. 2005; Dirani, Chamberlain et al. 2006). Corneal curvature shows heritability values of

0.71-0.95 in twin studies (Dirani, Islam et al. 2008; Klein, Suktitipat et al. 2009), with lower values reported in family studies of 0.16-0.57 (Biino, Palmas et al. 2005; Chen, Scurrah et al. 2007; Garoufalis, Chen et al. 2007). Corneal astigmatism is reported to show a significant genetic component with heritability values of 0.5-0.65 in twin pairs (Hammond, Snieder et al. 2001; Dirani, Chamberlain et al. 2006; Grjibovski, Magnus et al. 2006; Dirani, Islam et al. 2008), and there is some evidence that most of this is due to dominant genetic effects (Hammond, Snieder et al. 2001; Dirani, Islam et al. 2008). Family studies show corneal astigmatism heritability of 0.3-0.47 (Mash, Hegmann et al. 1975; Cagigrigoriu, Gregori et al. 2007), and heritability of corneal power has been shown to have a high heritability of 0.89 in parent-offspring pairs (Mash, Hegmann et al. 1975). Corneal thickness also displays a high heritability of 0.88-0.91 (twin pairs)(Zheng, Ge et al. 2008).

Twin studies have reported high heritability values for axial length ranging from 0.83-0.94 (Sorsby and Fraser 1964; Lyhne, Sjolie et al. 2001; Dirani, Chamberlain et al. 2006), with lower values in family studies of 0.20-0.73 (Biino, Palmas et al. 2005; Chen, Scurrah et al. 2007; Garoufalis, Chen et al. 2007; Paget, Vitezica et al. 2008; Klein, Suktitipat et al. 2009). Anterior Chamber Depth shows heritability values of 0.51-0.88 in twin studies (Lyhne, Sjolie et al. 2001; Dirani, Chamberlain et al. 2006) and 0.44-0.78 in family studies (Biino, Palmas et al. 2005; Chen, Scurrah et al. 2007; Garoufalis, Chen et al. 2007).

It appears anthropomorphic measurements such as stature and body weight also influence the development of refractive error. In twin studies, females with heavier body weight have been shown to be more likely to have a myopic refractive error (Dirani, Islam et al. 2008). Teikari found that taller males were significantly more myopic, but this was not found in females (Teikari 1987). Body Mass Index (BMI) also seems to play a part, when myopic and non-myopic twin partners were compared, BMI was significantly different between twin partners, with myopia being associated with higher BMI but again this finding was only found in males (Teikari 1987).

2.1.6. Myopia Family Studies

The inheritance of myopia within families has also been studied across a variety of populations, both between parents and children, siblings and more distant relationships (Krause, Rantakallio et al. 1993; Goss and Jackson 1996; Edwards 1998; Bullimore, Jones et al. 2002; Mutti, Mitchell et al. 2002; Biino, Palmas et al. 2005; Iribarren, Balsa et al. 2005; Wojciechowski, Congdon et al. 2005; Chen, Scurrah et al. 2007; Paget, Vitezica et al. 2008;

Klein, Suktitipat et al. 2009). The studies discussed below have somewhat differing results, however it should be noted that they have used different family types, age groups, ascertainment strategies, sample sizes, methodologies, definitions and variable types of data analysis. Generally, correlations of refractive error between siblings would be expected to be higher compared to those from parent-child comparisons, as siblings would be expected to share both genetic and environmental influences (Rose, Morgan et al. 2002).

Heritability values of myopia within families have been reported in numerous studies and vary between 0.2-0.62 (Biino, Palmas et al. 2005; Wojciechowski, Congdon et al. 2005; Chen, Scurrah et al. 2007; Paget, Vitezica et al. 2008; Klein, Suktitipat et al. 2009).

Numerous studies have shown a correlation between parental myopia and myopia development during childhood (Krause, Rantakallio et al. 1993; Goss and Jackson 1996; Kurtz, Hyman et al. 2007; Mutti, Cooper et al. 2007; Konstantopoulos, Yadegarfar et al. 2008). Fewer studies have reported no association (Edwards 1998). A family history of myopia seems to influence the final adult refractive error in some populations (Saw, Nieto et al. 2001), but not in others (Bullimore, Jones et al. 2002; Iribarren, Balsa et al. 2005). Evidence does suggests that the offspring of highly myopic parents develop myopia earlier in life (Liang, Yen et al. 2004), and that children born to myopic parents show faster progression rates (Saw, Nieto et al. 2001). There is also evidence that the rate of eye elongation varies dependent on paternal myopia. Children with two myopic parents show increased axial length and resultant myopic shift compared to children with no paternal myopia (Lam, Fan et al. 2008). Longer axial lengths were found even before children developed a myopic refraction, with emmetropic children having longer eyes if both parents were myopic compared to either one or no myopic parents (Zadnik, Satariano et al. 1994).

Studies in different populations have found that myopic parents are more likely to have myopic children, especially if both parents are myopic, with the prevalence being 30-43.6%, compared to 14.9-25% with only one myopic parent and the level decreasing to less than 10% if neither parent is myopic (Ashton 1985; Mutti and Zadnik 1995; Goss and Jackson 1996; Mutti, Mitchell et al. 2002; Ip, Huynh et al. 2007). Liang et al conducted a study dividing the degree of myopia into different groups (Liang, Yen et al. 2004); mild, moderate and high and investigated odds ratios for children developing myopia. This study found that children of parents with high myopia were much more likely to develop high myopia (OR of 5.47, 6.03 for one and two myopic parents respectively) than children of parents with either mild or

moderate myopia. High myopia was also shown to have a greater heritability than mild or moderate myopia. There was a weaker association observed between siblings, with one highly myopic sibling (OR -5.16). A family study by Farbrother et al (Farbrother, Kirov et al. 2004) investigated a UK cohort of probands with high myopia (≤-6.00D). The sibling recurrence risk (K_s) and sibling recurrence risk ratio (λ_s) were used to assess the risk of high myopia development in family members of the probands. Ks was found to be 10%, meaning 1 in 10 high myopes had a highly myopic sibling. However, it was found that in 40% of highly myopic cases, neither parent was myopic, and siblings were less likely to have high myopia (K_s~6%), compared to those who had one or two myopic parents. This study demonstrated that although high myopia seems to show a more definite genetic causation in some research (Liang, Yen et al. 2004); including genetic linkage studies. The results found by Farbrother et al, with a relatively low sibling recurrence risk and large number of cases without myopic parents suggests a more complex, multi-factorial aetiology.

Many studies have demonstrated a hyperopic shift in refraction with age, with a myopic shift in later age (65 to 70 years of age) due to lenticular changes (Lee, Klein et al. 2002; Guzowski, Wang et al. 2003; Fotedar, Mitchell et al. 2008). However, across generations there is a different trend for an increase in myopia.. Wu et al (Wu and Edwards 1999) investigated the prevalence of myopia across three generations in a Hong Kong population, and found myopia became increasingly prevalent in each new generation. Again it was found that myopic parents were more likely to have myopic offspring, but it was also found that the prevalence of myopia was also increasing in children where neither parent was myopic, suggesting substantial environmental influences in the most recent generation.

Guggenheim et al (Guggenheim, Pong-Wong et al. 2007) investigated a group of Singaporean schoolchildren aged 7-9, and looked at correlation of refractive error and ocular biometry measurements and environmental factors such as time spent involved in near work, and found that all of these factors showed significant correlations. Correlation of refractive error measurements between siblings was 0.447, suggesting that familial factors accounted for the majority of variation in the group studied. Of the 147 sibships studied, the odds ratio for myopia was 3.24. However, a strong environmental correlation was also found between siblings, making it difficult to identify if genetics or environment played a greater role.

Other studies have investigated the levels of myopia between siblings and cousins. One such study by Lee et al (Lee, Klein et al. 2001) found an odds ratio of 4.18 for myopia and 2.87 for

hyperopia between siblings which was higher than that found for the parent child risk which was 1.41 for myopia and 1.80 for hyperopia. The cousin risk was smaller again with 1.27 for myopia and 1.59 for hyperopia. In a truly inherited genetic trait it would be expected that the parent-child and sibling OR would be the same, with the cousin figures being less. The figures found in this study support an environmental element, in that siblings would have more environmental influences in common with each other than they would with their parents.

2.1.7. Refractive Error in Animals

Refractive error has been tested in a range of wild, domesticated and laboratory animals. Most animals including cats (Belkin, Yinon et al. 1977), monkeys (Young 1964), rabbits (Pak 1984), grey squirrels (McBrien, Moghaddam et al. 1993) show on average low hyperopic refractive errors. Refractive error and ocular development has been particularly important in animal models of myopia. Accurate estimates of average refraction and ocular parameters are important where refractive errors are induced, and ocular measurements are compared after form deprivation or lens induced myopia develops. Animal models including guinea pigs (Zhou, Qu et al. 2006), tree shrews (Norton and McBrien 1992), and non-human primates (Bradley, Fernandes et al. 1999; Graham and Judge 1999) show similar refractive development, being born with a moderate hyperopic refraction which shifts towards emmetropisation during early development

2.1.8. Refractive Error in Dogs

Since the late 19th century there has been interest in the refractive state of wild and domestic dogs. Many of the early studies found that a small proportion of domestic dogs showed a myopic refraction, compared to wild jackals, wolves and dingoes which showed emmetropic or low hyperopic refractive errors (Johnson 1901). Some early researchers reported levels of myopia of up to -3.00D (Nowak and Neumann 1987). The first extensive study conducted in the 1920s, which tested over 100 dogs found a greater variety of refractive errors with a range of +2 to -4.5D (Dubar 1920).

There have been several more recent studies investigating the distribution and prevalence of myopia in a range of dog breeds. Most studies have reported the average refraction to be emmetropic or in the low hyperopic range (Nowak and Neumann 1987). A German study investigating biometric parameters in conjunction with cataract surgery (Gorig 2000) (n=280)

reported the average refractive error to be nearly emmetropic with 38.6% of the dogs tested in the myopic range. However this may have been due to varying amounts of lenticular opacity (not reported). They also reported that larger breeds were associated with more hyperopic refractive errors, and that smaller breeds tended to be more myopic, which has not been reported in any other studies.

One study investigated the refractive error in 11 breeds of dogs (Murphy, Zadnik et al. 1992). The conclusion was that on average most breeds were slightly hyperopic. However, German Shepherds, Rottweilers and Miniature Schnauzers were found to be myopic (on average -0.86 DS, -1.77DS and -0.67DS respectively). Murphy's study (Murphy, Zadnik et al. 1992) also tested a group of German shepherd guide dogs, it was found in this population that the average refractive error was slightly hyperopic, suggesting that guide dogs with myopia exhibit characteristics which make them unsuitable for training (Murphy, Mutti et al. 1997). Labrador Retrievers were found to have an average refractive error of +0.63DS. Another study by Mutti et al (Mutti, Zadnik et al. 1999) concluded that like in humans, an elongated vitreous chamber is the cause of myopia in Labrador Retrievers and there was also a slight effect from lens thinning (Mutti, Zadnik et al. 1999). Murphy tested dogs with myopia to investigate the effect on visual function, and found that as in humans, the associated defocus results in a reduction in visual acuity (Murphy, Mutti et al. 1997). The implication was that dogs in service industries should be screened for refractive error. A more recent study (Kubai, Bentley et al. 2008) has supported previous findings, testing refractive error on 1440 domestic dogs, and found a slightly negative SER across all breeds of -0.05D, with a range of refractive errors of +6.00 to -6.00D. It should be noted that in the study of Kubai et al all available dogs within the veterinary facility were tested, from 0.1 to 15 years of age. The inclusion of older animals may have biased the average refractive errors due to lenticular changes.

2.2. Aims of Chapter Two

2.2.1. Aim 1: To measure refractive error and collect DNA samples in a large number of related dogs in order to identify those which are myopic

Previous studies have identified that various dog breeds show a range of refractive errors, with a significant proportion of dogs being myopic. The dog is the only species which shows naturally occurring myopia (Kubai, Bentley et al. 2008). The first aim was to test a large pedigree of purebred dogs to get a more accurate estimate of refractive error across a range of generations and litters. This was a precursory step to statistically testing if refractive error in dogs is inherited. Myopia has shown to show a strong genetic component in human families, but this had never before been demonstrated in a non-human species. We chose a large pedigree of Labrador Retrievers because they are the only breed of dog in which myopia has been shown to be axial in nature (Mutti, Zadnik et al. 1999).

2.2.2. Aim 2: To construct large pedigrees to identify the pattern of inheritance of myopia

The first step in any familial study of disease is assessing statistically if the disease occurs due to inheritance (genetic components) or due to environment (environmental and spurious causation). Familial aggregation and heritability calculations are a way of detecting if a trait is inherited or is more likely to have been caused by environmental factors. Familial aggregation involves studying the pattern of a trait within a family, without identifying a specific genetic model (segregation analysis). Heritability is the variance of a phenotype due to genotype. Once Aim One was completed, the pattern of refractive error was analysed using variance component modelling over the entire Labrador Retriever pedigree. Due to the pedigree structure and high level of consanguinity a mode of transmission (ie. dominant/recessive) could not be tested.

This chapter outlines the literature and background supporting aims one and two and then outlines the methods and results used in the familial aggregation portion of the project. This chapter describes the recruitment of subjects used throughout the project (aims 1-4), methods of refractive error measurement and the statistical analysis which supported continuing to aims three and four. It should be noted that there were two groups of Labrador Retrievers

phenotyped in this project; chapter two discusses only the large pedigree used for familial aggregation analysis. This portion of the project has been published in Investigative Ophthalmology and Vision Science (Black, Browning et al. 2008).

2.3. Methods

2.3.1. Recruitment of animal subjects

This project is a continuation of previous (unpublished) studies (Corbett 2002; Black 2004; Collins 2004) in cooperation with Guide Dog Services (GDS), a division of the Royal New Zealand Foundation of the Blind (RNZFB). GDS has a large breeding colony based in Manurewa, Auckland, New Zealand. The GDS breeding colony is composed of a number of breeds, most dogs being Labrador Retrievers. Other breeds include German Shepherds, Border Collies, Poodles, Boxers and Australian Shepherds. Labrador Retrievers were chosen for this study because they are the only breed that has been shown to have naturally occurring vitreous-chamber based (axial) myopia (Mutti, Zadnik et al. 1999) making it comparable to the human condition. We have previously worked with the GDS colony to investigate the correlation of refractive error and vision, behaviour and refractive error (Collins 2004) and to test the canine contrast sensitivity function (Black 2004).

2.3.2. Guide Dog Training

From birth to qualifying as a working Guide Dog, puppies go through several stages of training. We tested dogs involved in all these stages, but most dogs were in the training stage between puppy development and qualifying as guide dogs.

Puppies are born in the breeding colony at Manurewa and stay there until six weeks of age having had initial vaccinations, veterinary and ocular checks. They are then placed with "puppy walker" families, the majority being within the greater Auckland area, but some are placed in other areas around New Zealand, where they stay until they are 12-18 months of age. Within the first year of life their progress is monitored by a development manager, who meets the puppy walker monthly to check on the dog's progress. Dogs are also periodically returned to the centre at Manurewa where they have regular health checks. During the first 12-18 months, they live in a domestic environment, but this differs somewhat from that of a normal pet dog. All puppies are kept inside with regular time outdoors. Puppy walkers are encouraged to take the puppies with them to work and for other tasks such as shopping and going out for meals. Puppies are allowed into all public places and onto public transport. At about 18 months of age the puppies are assessed in terms of health and temperament. Those unsuitable for guide dog training are withdrawn at this point, either being adopted by their puppy walkers or going into parallel training i.e. disability dogs, hearing dogs, bomb dogs,

customs etc. On being accepted to Guide Dog training they are placed with a trainer based at Manurewa who supervises their training for 3-6 months (it was during this time, that the dogs were easily accessible for refractive error testing). After this part of training is complete, a decision is made about whether the dogs will continue to become guide dogs, breeding stock, are unsuitable – so are withdrawn, or are to be sold overseas. Once a dog has passed training, they are matched with a client in terms of temperament, personality and physicality. They are then trained with the client by a regional manager who continues to monitor the dog's health and behaviour. Breeding dogs are housed with guardians but have regular contact with the centre at Manurewa, and have annual eye checks. The majority of breeding dogs were tested in this study because it was particularly important to determine their phenotype for the familial aggregation study.

Guide Dog Services also has information regarding those dogs which have been withdrawn and placed with families. These owners were contacted to get approval for their involvement in the study.

Ethics approval was granted through the Auckland Animal Ethics committee (AEC/08/2005/R407 – The Genetic Basis of Myopia in Dogs). Owners were informed of the purpose of the study and were asked for permission to have their dogs participate. Owners were given the results of the refractive error testing on request.

2.3.3. Control dogs recruited from outside the GDS colony

The third aim of this project was to investigate the underlying genetic cause of refractive error in Labrador Retrievers. Thus, a control population was needed that was not myopic. The main criteria for this population were that the dogs were not myopic and that they were as unrelated to the GDS population as possible. The criteria for myopia in this case was a refractive error equal to, or more minus than -0.50D spherical equivalent. All of these dogs were obtained from other Labrador Retriever breeders from the North Island of New Zealand. Proof of breed was again ascertained by checking records with the New Zealand Kennel Club as discussed further in Chapter Three.

At contact appointment
Refractive Error Measured under cycloplegia
DNA sample collection
Phenotypic Characteristics e.g. confirmed coat colour, any ocular anomalies
Other Data collated from breeding records
Relevant health history of dog, parents and littermates
5 generation pedigree
Outcomes of all ophthalmological checks
Environmental Data e.g. birth weight
Current locations of Sire, Dam and Littermates

Table 1 Information collected for each dog in the study

2.3.4. Measurement of Refractive Error

One of the initial challenges was determining the best method for fast and reliable measurement of refractive error in dogs. Previous studies of canine refractive error have generally used cycloplegic retinoscopy (Murphy, Zadnik et al. 1992; Mutti, Zadnik et al. 1999; Corbett 2002; Black 2004; Collins 2004; Kubai, Bentley et al. 2008). It was hoped that an automated method could be utilised to reduce experimenter error and potential bias.

2.3.5. Auto-Refraction

The first method trialled was cycloplegic auto-refraction. A Welch Allyn Suresight auto-refractor (Welch Allyn, Buckinghamshire, UK) was selected as it has been validated as a reliable and successful method in human infants (Cordonnier and De Maertelaer 2004). The only bias reported is that it tends to give a slightly higher myopic prescription and is more accurate in reading myopic rather than hyperopic prescriptions (Cordonnier and De Maertelaer 2004). The Suresight also had the advantage of being hand-held and portable with a testing distance of 35cm.

After the auto-refractor had been trialled in a number of dogs of different breeds, several problems became apparent. Dogs have a natural instinct to turn their head away from direct confrontation, so when faced with a device at 35cm, they tended to turn their head/eyes to the

side. Also, the target on the Suresight is a very small light, and it was difficult to ascertain if the dog was looking exactly in the direction of the target. The Suresight also required that the subject kept their head position straight and their gaze steady. In combination these factors led to a high rate of measurement error. When compared to retinoscopy on the same animal, rates of astigmatism in particular were found to be much higher, which gave a more negative spherical equivalent refraction (SER)(sphere + ½ cyl) than retinoscopy. The Suresight has an inbuilt reliability measure ranging from one to nine. The manufacturer suggests that five or greater is required to give a reliable estimate of refractive error, but in dogs it was difficult to get reliability measurements within the correct range. Where an acceptable reliability measurement was not attained in the first measurements, additional measurements were attempted. However, it was often difficult to get more than one measurement due to inattention of the animals. Where more than one measurement was taken, the measure with the highest reliability value was reported. The Suresight was attempted on both eyes of 38 dogs of variable breeds (Labradors, Poodles, Golden Retrievers and German Shepherds). However, only results for Labrador Retrievers are reported. Of the 33 Labrador Retrievers tested, 30 dogs had successful measurements from both eyes (and 1 eye of 3 dogs). Only a small proportion of measurements gave acceptable reliability measures. Auto-refraction measurements were not included in analysis as they gave higher cylinder readings, thus creating artificially high levels of myopia when SER was calculated.

2.3.6. Photo-Refraction

Photo-refraction (eccentric) measures the refractive error of the eye by analysing the light pattern across the pupil (Roorda, Campbell et al. 1997). The eyes are photographed in the plane of the pupils which are illuminated by an array of LEDs located in the plane of the camera aperture but eccentric to it. Depending on the level of ametropia the light pattern in the pupil will either be dark or fill a variable area of the pupil. The amount of ametropic defocus can be measured by the height of the light crescent within the pupil, with greater defocus creating greater light spread across the pupil. Lights from different eccentricities are used as this prevents the entire pupil being filled with light when the level of retinal defocus is high. The main variables influencing measurements are camera to subject distance, pupil size, eccentricity of light source to camera aperture, and degree of defocus in the subject (Howland and Sayles 1985).

A simple photo-retinoscope was built and tested on a small number (24) of dogs (see Figure 1). The design was based on a previous study which successfully measured refractive error in a variety of animals (Schaeffel, Hagel et al. 1994). The photo-retinoscope was built using 22 1mm yellow LED's with four rows of 4 LED's and one row of 6 LED'S. The spacing of the LED's was calculated so that the array was equally spaced, with the total diameter equal to half of 49mm, the diameter of the camera lens.

Dogs were placed at 1.5m from a video camera in a sitting position. They were filmed for between 30-60 secs, and were 'called' to get them to look towards the camera. The photo-retinoscope was attached to the lower half of the video camera lens. Still images were then extracted from the footage using Windows Movie Maker (Microsoft Corporation, USA), for further analysis. Images in which both pupils were illuminated, the corneal reflex was central and the head was in a straight-ahead position were saved. Saved images were then opened in Image J software, the pupil was selected using the circle tool and the greyscale pattern was analysed across the pupil. Photo-refraction data was not used in familial aggregation analysis. After unsuccessfully trialling the photo-refractor on a small sample of dogs, it was not continued as a method of attaining refractive errors. Photo-refraction was not a practical method when testing dogs in a variety of locations as it was not always possible to get the testing location dark enough to get a satisfactory pupil reflex, the equipment was cumbersome and took time to setup and some dogs were not able to be tested inside.

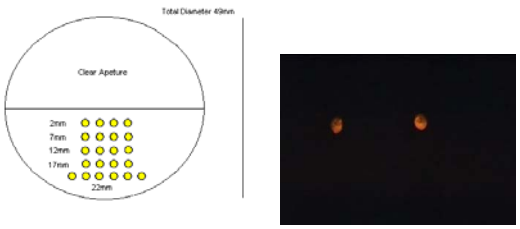


Figure 1 Diagram of photo-retinoscope design (left). Photo showing pupil reflexes with photo-retinoscope (right)

2.3.7. Cycloplegic Retinoscopy

Cycloplegic retinoscopy is a common measurement technique in studies of refractive error involving infants as well as other animals (Twelker and Mutti 2001; Davitt, Dobson et al. 2005; Kubai, Bentley et al. 2008) and retinoscopy proved to be the most efficient and reliable

method when testing dogs. Measurements could be taken quickly in a variety of locations, with dogs in different positions and in a range of different light levels. Two independent retinoscopists (the author and another registered optometrist) tested each animal where possible. Two drops of Mydriacyl (Tropicamide 1.0%)(Alcon Laboratories) were instilled into each eye, five minutes apart, 30 minutes prior to refractive error testing. Tropicamide has previously been shown to provide adequate cycloplegia in studies of children (Egashira, Kish et al. 1993; Twelker and Mutti 2001). Attaining full cycloplegia was not as critical as in human studies, as dogs are estimated to have a maximum of only 2-3 dioptres of accommodation (Hess 1898). A previous study measuring canine refractive error found no significant difference in refraction with and without cycloplegia (Nowak and Neumann 1987). Refractive error was measured along the two major meridians with a lens bar with 0.50 dioptre steps. Refractive error was taken as SER for each eye. For each dog, the refractive error used in the statistical analyses was the average of the right (RE) and left eye (LE) SER's.

2.3.8. Inclusion Criteria

1. Dogs were required to be a registered purebred Labrador Retriever with the New Zealand Kennel Club, an organisation which ensures purity of Dog breeds. As cross-breeding (e.g. between Golden Retrievers and Labrador Retrievers) is common it was important to have multi-generational records to prove pedigree, as these crosses are difficult to detect purely by physical characteristics. For proof of breed the New Zealand Kennel Club requires a five generation pedigree and the dog to be registered with an international kennel club registry.

2. For the aggregation analysis age limits of 1-8 years were applied. The aim was to ensure that refractive error development was stable and that the majority of growth was complete. One year was chosen as this is equivalent to 18 years of age in humans. The upper age limit of 8 years (equivalent to 56 years of age in humans) (Waters, Patronek et al. 1996) was chosen to exclude dogs with potential lenticular changes that might have led to an increased level of myopia.

2.3.9. Exclusion Criteria

1. As animals were cyclopleged with 1.0% tropicamide (Mydriacyl, Alcon Laboratories), they were not tested if they had a history of previous ocular allergies.

2. Refractive error measurements were not included where opacities were observed in the lens or ocular media with retinoscopy.

3. Animals were excluded if they had systemic disease such as epilepsy or arthritis which could influence refractive error readings due to the nature of the disease or associated medications.

4. As dogs were being tested without sedation, they had to be cooperative in order to get accurate refractive error readings. Dogs were excluded if they exhibited aggressive behaviour or if retinoscopy caused them distress.

5. All dogs were checked by a veterinary ophthalmologist at least once. Ophthalmology records were inspected and dogs with any ocular pathology such as retinal folds, cataracts, small optic nerves, hyphaema or corneal dystrophies were excluded from the study. Any dogs with a history of ocular trauma or surgery were also excluded.

2.3.10. Database and Pedigree Construction

A database of the entire GDS colony of 1387 dogs was initially constructed (by the author) using Breedmate Pedigree Software (Wild Systems 2007, Australia). Breedmate is a software package designed for breeders of purebred dogs/cats. It allows relationships to be easily assessed through the construction of pedigree/descendants charts. There are also fields for physical traits and genotypes. Fields for refractive error measurements (autorefractor, retinoscopy and mean sphere) were added for this study.

Information for the database was obtained from paper breeding records. There were approximately 128 breeding dogs in the GDS colony (both past and present). Records were double checked to ensure accuracy by checking records of both sire and dam, and by cross checking with pedigree records held with the New Zealand Kennel Club which registers all purebred dogs in New Zealand (www.nzkc.org.nz). Puppies that never entered the guide dog training programme were not included in the database as GDS does not follow up these dogs. Deceased animals were included, as some of these dogs had been tested previously

(approximately 30 dogs). For each animal, data fields included Name, Gender, Date of Birth, ID number, Sire, Dam, Breed, Coat Colour, Status within the Guide Dog program, health history (cancer, epilepsy, allergy etc), ocular history, refraction and spherical equivalent refraction. All breeds were entered in the 1st version of the database, so that the entire colony was represented. This included all animals from breeding records.

Pedigree construction revealed one large family descended from a single stud dog. This dog was known to be myopic (from a previous study (Corbett 2002)), and to have a produced a moderately myopic litter with a myopic female. As of the end of 2008 this family consisted of a total of 478 individuals from 72 litters. The initial part of the project aimed to test as many members of this pedigree as possible.

Cranefoot (Makinen, Parkkonen et al. 2005), pedigree drawing software was used for pedigree diagram construction as it allowed visualisation of the inbreeding and mating loops within the pedigree. A text file was created within Microsoft Excel with columns specifying family ID, individual dog ID, Sire, Dam and phenotype. Cranefoot then generated the pedigree diagrams as .pdf and .postscript files.

Refractive error and pedigree information were also entered into excel 2003/2007 (Microsoft Corp). Excel was used to keep track of which dogs had been tested, DNA collection procedures and to construct data for statistical analysis.

2.3.11. Sampling Strategy

As described earlier the initial two aims of the study were to test a large number of related Labrador Retrievers and to use statistical analysis (familial aggregation) to ascertain if refractive error in this family was inherited or more likely caused by environmental influences. To do this we chose one large family from the GDS breeding colony (described above). From this family we aimed to sample all parents (breeding animals) and at least two dogs from each litter. This strategy allowed all possible relationships within the pedigree to be sampled. Most animals were still available for testing. However, the earlier branches of the pedigree represented litters born in the early to mid 1990s, so were outside the inclusion criteria for age. Many of the breeding animals in the early litters were also outside the age limits. However, some of these animals had been tested previously in earlier projects with GDS so their phenotypes were included in the analysis if they met the inclusion criteria when they were tested.

2.3.12. Data Analysis

2.3.12.1. Comparison of Refractive error measurement methods

The repeatability between the different methods of refractive error measurements was assessed using Bland-Altman analysis (Bland and Altman 1986) ((cycloplegic retinoscopy versus autorefraction) and between the two retinoscopists). This type of analysis looks at the level of agreement between clinical measurements rather than just the relationship between them, as would be tested with correlation coefficients. Bland Altman analysis can be used to test the agreement between different types of tests, in this case retinoscopy versus autorefraction and between clinicians using the same clinical test.

2.3.12.2. Refractive Error Analysis – Auto-Refraction

To average sphero-cylindrical components of refractive error measurement with cycloplegic autorefraction, measurements were broken down into components of sphere, cylinder and axis. Each of these results was converted into power vectors represented by M, J0 and J45 notation using Fourier analysis (Thibos, Wheeler et al. 1997). M represents the mean sphere; with J0 and J45 representing the cylinder as the two components of a Jackson Crossed Cylinder (JCC). As each of these values represents a vector, the values can be summed, averaged and analysed individually and then combined and converted back to standard clinical notation to give an average sphero-cylindrical value. Excel was used to calculate power vectors, with equations previously described by Miller et al (Miller 2009). This technique was not repeated for cycloplegic retinoscopy as the level of astigmatism was negligible.

2.3.12.3. Familial aggregation analysis

Most software packages that calculate segregation analysis (see glossary) and heritability (see glossary) cannot cope with inbreeding loops or mating clusters (one individual having offspring with more than 3 partners) as they are designed for human populations where this is generally not found. Segregation analysis was initially attempted with software package SAGE (statistical analysis for genetic epidemiology) as had been done in previous studies of canine disease segregation analysis studies (Cargill, Famula et al. 2004; Janutta, Hamann et al. 2006). However, using SAGE resulted in having to break down the pedigree into nuclear families, meaning that more distant relatedness was lost.

Finally, MORGAN software V2.8.2 (University of Washington, Seattle, WA) was used to analyse the data because it permitted analysis of large pedigrees with multiple mates (more than three mates) and consanguinity, which characterized our sample. Our analysis accounted for all known relationships between the phenotyped dogs and their shared ancestors. MORGAN allowed analysis of variance between genetic and environmental components, but did not allow a mode of transmission to be assessed (segregation analysis – see glossary).

The pedcheck program of MORGAN was used to check for relationship errors within the pedigree such as gender errors or parents born after offspring. Both sire and dam had to be specified in pedcheck to allow accurate kinship coefficients to be made between all relation pairs, where only one parent was known, a second parent was generated. Of the 134 animals included 25 were founders and had no sire or dam listed. The multivar program of MORGAN was used to obtain maximum likelihood estimates for additive polygenic models of quantitative traits by the expectation-maximization (EM) algorithm. We analysed refractive error as a quantitative trait, with three fixed effects; gender, litter size (1-13) and coat colour (black or yellow). We included gender as there is some suggestion of a gender effect in human studies (Chen, Scurrah et al. 2007). Although there is no previous literature to suggest that either coat colour or litter size would be associated with myopia in dogs, both are phenotypic descriptors that do not alter with age. Litter size has been shown to influence birth weight and term of gestation which has been previously associated with refractive error development in human (Varghese, Sreenivas et al. 2009). All fixed effects were available for most dogs and were included where known; they were coded as 0 if unknown. Gender was known for all dogs. Coat colour and litter size were unknown for some of the older founder animals that had come from outside colonies. Coat colour was unknown in 5 dogs, and litter size was unknown in 18 dogs. The kin program within the MORGAN package was also used to calculate inbreeding coefficients between individuals of interest. Narrow sense heritability was calculated using variances computed by multivar for the best fitting sub-model only.

The additive polygenic model used by multivar (Thompson and Shaw 1990) is $y = \mu + z + e$, where y is the quantitative trait (in this case refractive error, defined as spherical equivalent refraction), μ is a vector of fixed effects, z is a vector of additive genetic effects, and e is a vector of residual/environmental effects. Normal distributions were assumed for z and e. The elements of e are assumed to be independent with common variance. The elements of z have correlations proportional to the coefficients of kinship. The model is fit with the multivar program using the EM algorithm, which is an iterative approach to finding the parameter

values that maximize the model likelihood. Stability of parameter values was typically achieved by 50 iterations although we always used 200 iterations which is the default for multivar.

A histogram of refractive error distribution within the sample showed that it was left-skewed. Approximate normality was obtained by the transformation SQRT (1.65 - SER). This transformation was chosen to first obtain positive right-skewed values (the largest untransformed SER value was +1.65D), and then to reduce the skew-ness through use of the square-root transformation. The transformed values were used as the quantitative trait in subsequent analysis with the multivar program. We also ran the analysis using a 'normal deviates' transform (Legendre 1998) of the refractive error distribution. Litter size ranged between 1 and 13 puppies, and was coded as small (litter size between 1 and 7) or large (between 8 and 13).

In order to test for statistical significance of the fixed and genetic effects, nested models were compared. For example, we compared the full model with genetic effects, gender, coat colour and litter size to sub-models, in which each one of these effects was removed in turn. The null hypothesis was that the sub-model was as good as the full model at explaining the variability of the trait. Twice the difference in log-likelihoods from the two models was compared to a chi-square distribution with degrees of freedom equal to the difference in the number of estimated parameters between the two models. As each of our fixed effects had two possible states (apart from missing values) the degrees of freedom was equal to one in each case. If the resulting p-value was significant ($p < 0.05$), the null hypothesis was rejected, and we concluded that the variable that was removed from the full model to create the sub model was statistically significant in helping to explain the variability of the trait.

2.4. Results

2.4.1. Subject Recruitment

A total of 182 dogs were tested from the GDS colony in total, 134 of these animals were included in the familial aggregation analysis. The additional 66 dogs were tested after the analysis was complete and were from younger generations. The remainder of this chapter will discuss only the subgroup which were analysed. Chapter three will discuss the full pedigree and refractive status.

At the time that familial aggregation analysis was completed the pedigree consisted of 407 dogs from 64 litters. Refractive error measurements were made in 116 dogs; however one of the requirements of the software used was that both parents of all animals had to be listed, meaning that 134 dogs were included in analysis. Although refractive errors were not available for the additional 18 dogs, other fixed effects including gender, coat colour and litter size were available for most dogs. This pedigree is shown in Figure 2.

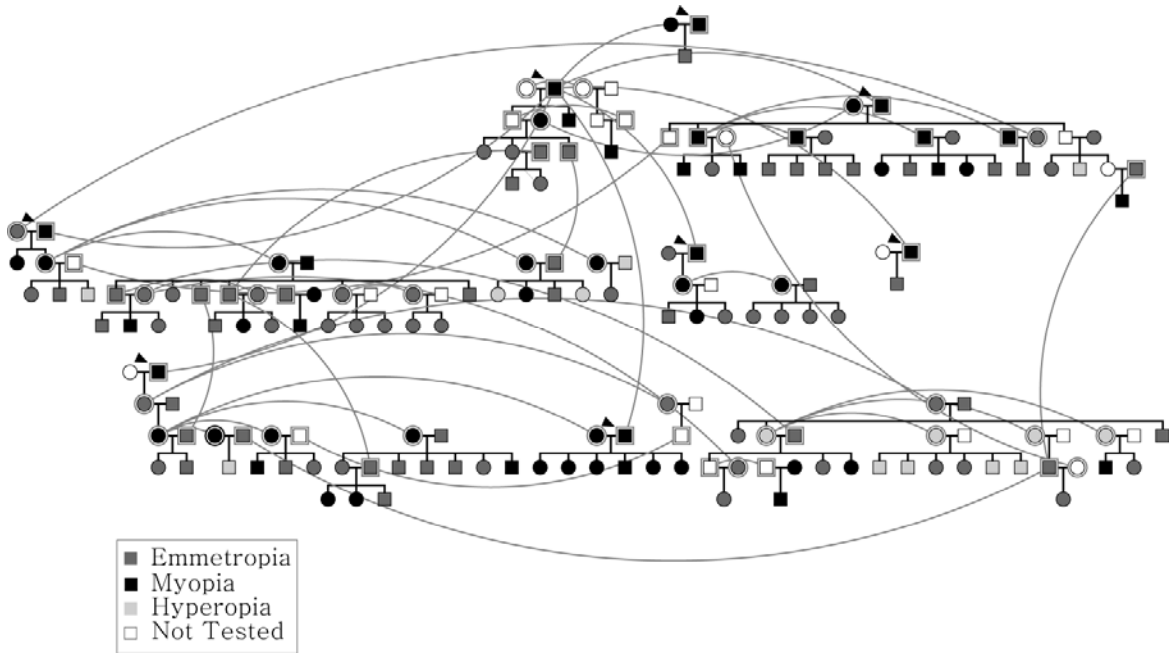


Figure 2 Pedigree Diagram of family phenotyped in familial aggregation analysis. The proband is marked by an arrow. Individuals may occur more than once in the pedigree, if they are parented more than one litter (indicated by curved lines).

2.4.2. Location of Testing

Of the total number of dogs tested from the GDS pedigree, 66 were tested at the GDS colony (including 11 who attended an organised day for adopted dogs), 6 were tested at the home of the animal within the Auckland Region and 32 were tested at other homes within New Zealand. The locations of the animals tested within New Zealand are plotted in Figure 3. Refractive error measurements were available for 12 dogs from previous research projects during (2001-2003).

Figure 3 Map of New Zealand showing locations of dogs tested. Squares are dogs from the GDS colony and circles are dogs from other New Zealand breeders (described in Chapter 3). The dogs tested from the GDS colony within the Auckland region are not included on this diagram.

2.4.3. Methods of Refractive Error Measurement

2.4.4. Auto-Refractor

The auto-refractor (Welch Allyn Suresight Autorefractor) was used to measure refractive error in 33 dogs (63 eyes). For all measurements the average SER was +0.16D (±1.30D, n =63)(SER range +2.99 to -3D). The average dioptric value of astigmatism was -0.89DC±0.67 (range -0.12 to -2.12DC). The average anisometropia was +0.33D±0.93D. Three dogs were only able to have one eye measured with this technique. The average reliability value indicated by the autorefractor for these measures was 4.26±0.95. An acceptable reliability measure (specified by the manufacturer as ≥5, on a scale of 1-9) was found in 20% of measurements. With auto-refraction, 53% were emmetropic, 26% were myopic and 21% were hyperopic (average of SER from both eyes).

Of the 12 measurements with acceptable reliability, average SER was +0.34D (±0.94D, n=12) with a range of +1.18D to -2.31D. Average anisometropia was 0.50D±0.76D. Average astigmatism was -0.68±0.54DC. Emmetropia was present in 75% of the sample, myopia in 8% and hyperopia in 17%.

2.4.5. Power Vector Analysis

The average sphero-cylindrical prescription (sphere (DS)/cylinder (DC) x axis (degrees)) for the RE was 0/-0.25x176 and the LE was -0.25/-0.25x50, following conversion from average V2C values (M, J0, and J45) back to C2V to give average values in sphero-cylindrical form (clinical notation).

Average J0 and J45 values were calculated separately for the R and L eyes. J0 was negative (-0.12) for the RE, indicating the axis was more against the rule (ATR) or vertical. J0 for the LE was positive (+0.03) indicating the axis was more with the rule (WTR) or horizontal (Huynh, Kifley et al. 2006).

J45 values were positive in the RE (+0.02) and negative in the LE (-0.14). This suggests that the RE axis was oblique sitting at approximately 135°, although as the value is close to zero, it would suggest that the axis sits closer to vertical. The negative value found for the LE suggests that the axis is also oblique, at approximately 45°, and as the value is greater than the RE, the LE axis is relatively more oblique.

2.4.6. Comparison of Refractive Error Methods

A paired t test showed no significant difference between AR and retinoscopy (p=0.12). However, Bland Altman analysis indicated that the level of agreement between auto-refraction and retinoscopy were well outside normal clinically acceptable repeatability. The lower limit of agreement was -1.91D and the upper level of agreement was 2.46D (±2SD). These results indicate that for 95% of comparisons, a measurement made with one method (i.e. retinoscopy) would be expected to lie between 1.91D lower or 2.46D higher than with the second method (auto-refraction), meaning that there could be up to 4.37D difference expected between SER measures with auto-refraction and retinoscopy. This is illustrated in Figure 4, which also would suggest that there is slightly more variability when measuring myopia in comparison to hyperopia, which is represented by the negative correlation -0.27.

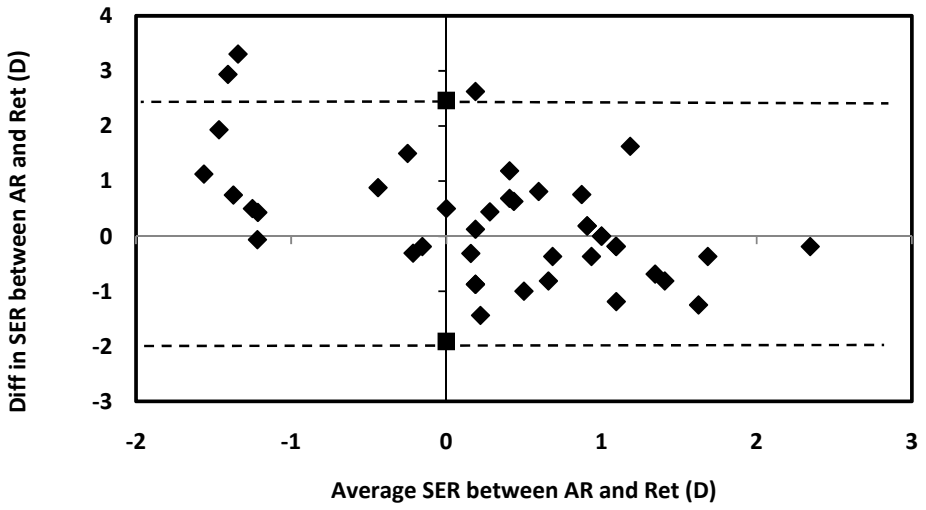


Figure 4 Bland Altman plot showing the average reading and difference between two clinical measurements of refractive error in dioptres (D); Cycloplegic retinoscopy (Ret) and Auto-Refraction (AR). Dashed lines represent upper and lower levels of agreement (±2 SD)

2.4.7. Retinoscopy

Following initial attempts at measuring refractive error with auto-refraction and photorefraction, cycloplegic retinoscopy was chosen as the most reliable method as well as being a common method in human studies of infants and children (Twelker and Mutti 2001; Davitt, Dobson et al. 2005). Cycloplegic retinoscopy was used to test all 116 dogs, with two retinoscopists giving independent results in the majority of cases.

Bland Altman analysis was used to test for repeatability between the two retinoscopists (Figure 5). For 95% of comparisons it was found that the two retinoscopy measurements were within ±1.10D of each other. Linear regression showed 0.06 and 0.02 correlations for the RE and LE respectively, suggesting minimal range effects.

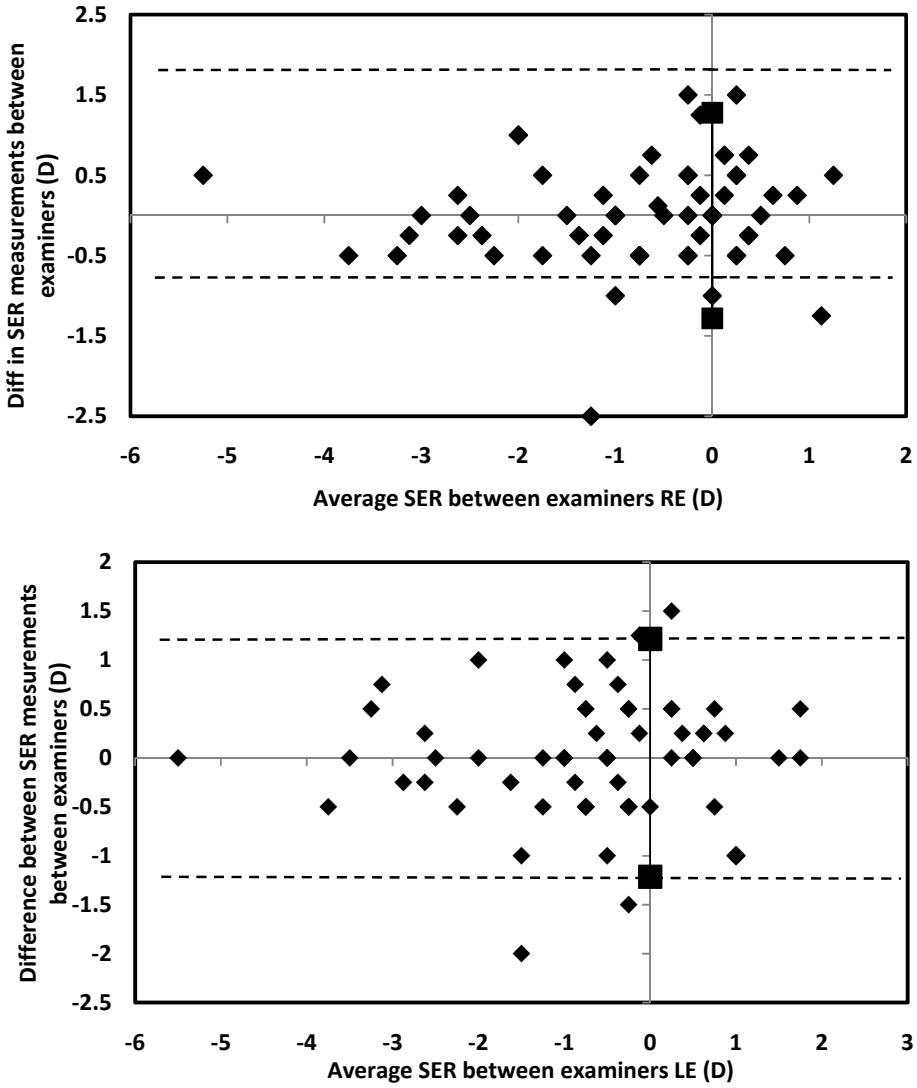


Figure 5 Bland Altman plot showing the average reading and difference between the two retinoscopy measurements of refractive error in dioptres (D). Dashed lines represent upper and lower levels of agreement (±2 SD)

2.4.8. Distribution of Rx in sample

The distribution of refractive error within the sample (Figure 6) shows a left-skewed curve, with a peak around emmetropia. There is an almost bimodal distribution with a second peak

around -2.50D. Of the 116 Labradors tested, the average refractive error was -0.41D (SER) with a range of +1.65D to -5.38D. The average difference in SER between the right and left eye was -0.13D, with anisometropia of ≥1.00D present in seven dogs (range 1 to 2.05D). There was good agreement between the SER found in the right and left eyes, with an average difference of 0.13D (R=0.963, after results were averaged between two retinoscopists). Emmetropia was present in 60% (SER -0.49D to +0.99D) of the GDS sample, 31% were myopic (SER ≤-0.50D) and 9% were hyperopic (≥ +1.00D). Of the 36 dogs that were classified as myopic 25% exhibited myopia ≥ -1.00D, 19% were -1.00 to -1.99D, 31% were -2.00D to -2.99D and 25% were ≤-3.00D. The range of refractive error in this family was +1.65D to -5.37D. The level of astigmatism was generally low in the population (taken as the difference between the horizontal and vertical meridians). The average difference between the two major meridians was 0.028D. Clinically significant astigmatism (>0.50DC) was present in 9 eyes from six animals (3.8%), and astigmatism of ≥-1.00DC was found in 3 eyes from two animals (1%). The range of astigmatism was -0.50 to -2.60DC. Power vector analysis was not repeated in results from cycloplegic retinoscopy, due to the minimal number of astigmatic animals. It should be noted that levels of astigmatism of less than 0.50DC were difficult to detect as the lens bar only graduated in 0.50D steps. One dog was antimetropic.

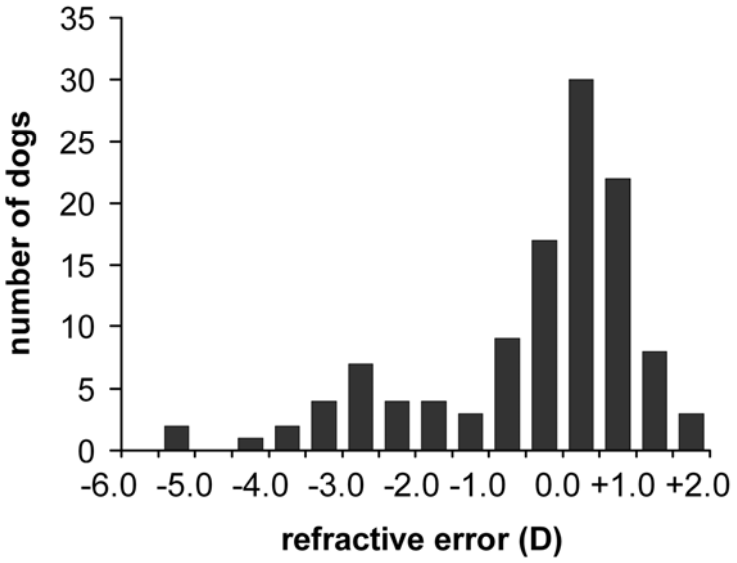


Figure 6 Histogram showing distribution of refractive error (average SER for both eyes) in the pedigree tested for familial aggregation analysis (n=116)

2.4.9. Age and Refractive Error

Although previous studies have demonstrated an increase in the level of myopia with increasing age in Labrador Retrievers (Kubai, Bentley et al. 2008), this is not demonstrated in the data in Figure 7, which shows no significant (Pearson R=0.0047, p=0.52) correlation between age and refraction. The average age of testing was 3.91 years (range 1-8).

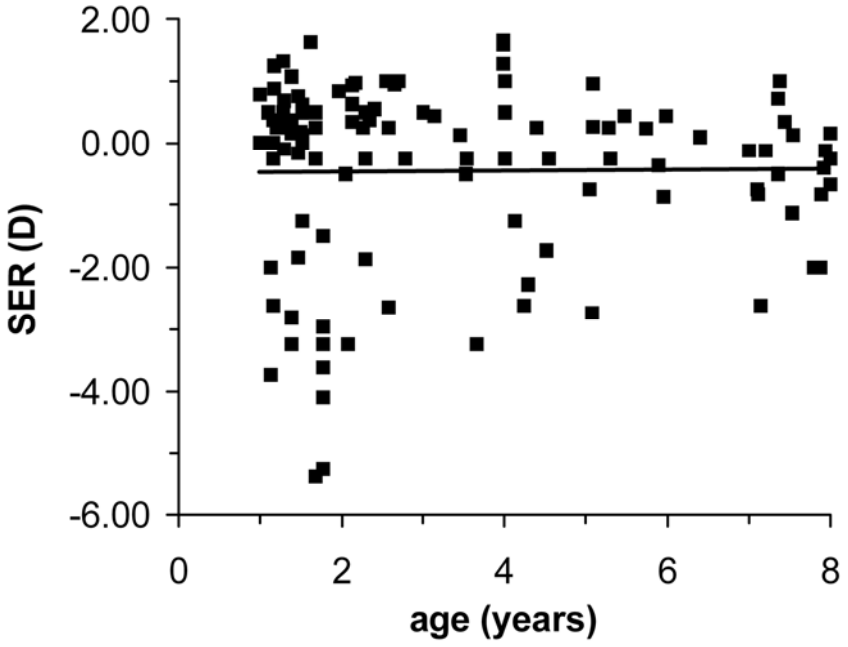


Figure 7 Scatterplot illustrating the relationship between age when tested and subsequent SER (average of R and L eyes). Age is restricted from 1 to 8 years.

2.4.10. Familial Aggregation Analysis

The statistical significance of fixed effects (gender, coat colour and litter size) and genetic effects was tested by comparing the full model (with genetic and all fixed effects) to models with one of these effects removed (see Table 2). Litter size and additive genetic effects were significant (p = 0.0013 and p = 0.000093, respectively), while gender and coat colour were not significant (p = 0.20 and p = 0.15, respectively). Similar results (in terms of statistical significance of each effect) were obtained by comparing a null model (with residual/environmental effects only) to models with only one of the fixed or genetic effects added. Analysis using the normal deviates transform confirmed the significance of litter size and additive genetic effects.

The final model, with litter size and genetic effects, can be described by the equation:

$$f(y) = \mu + \alpha_1 1\{\text{small}\} + \alpha_2 1\{\text{large}\} + z + e$$

where y is the SER, f is the transformation to improve normality f(y)= SQRT (1.65 - y); μ is the overall mean; 1{small} is one if the individual comes from a small litter and zero otherwise, while 1{large} is one if the individual comes from a large litter and zero otherwise (each individual comes from either a small or a large litter, so 1{small} and 1{large} always sum to one); α1 and α2 give the effects of small or large litter size; z is the additive genetic effect, which has a normal distribution with mean zero and variance σg^2 (with correlations between individuals being described by the matrix of kinship coefficients); and e is the residual/environmental effect, which has a normal distribution with mean zero and variance σe^2. The fitted values for these parameters were: μ = 1.218, α1 = 0.112, α2 = -0.062, σg^2 = 0.128, σe^2 = 0.118. There were 38 dogs from small litters and 68 from large litters and the variance for litter size was 0.007. Thus total variance was 0.128 + 0.118 + 0.007 = 0.253, and narrow-sense heritability was 0.128 / 0.253 = 0.506 (computed from the best fitting model only).

The overall variance after accounting for litter size was split approximately equally between additive genetic variance and residual/environmental variance. Thus, genetic and undefined residual/environmental factors played approximately equal roles in determining refractive error in this family. Litter size made a much smaller contribution to refractive error. Dogs from small litters had an average transformed trait value of 1.218 + 0.112 = 1.330, which

corresponds to SER = 1.65 – 1.332 = -0.119D. On the other hand, dogs from large litters had an average transformed trait value of 1.218 – 0.062 = 1.156, which corresponds to SER = 1.65 – 1.1562 = 0.314D. Thus the model indicated that dogs from large litters had refractive errors that were on average 0.43D more hyperopic than dogs from small litters.

Model	Comment	log lik.	chi-sq.	P-value
G + S + C + L + E	Full model	-66.50	N/A	N/A
S + C + L + E	Test for additive genetic effect	-74.14	15.28	9.3 x 10-5 *
G + C + L + E	Testing for effect of sex (gender)	-67.31	1.62	0.20
G + S + L + E	Testing for effect of coat colour	-67.56	2.12	0.15
G + S + C + E	Testing for effect of litter size	-71.64	10.28	0.0013 *

Table 2 Models used to test for statistical significance of variables, with log likelihoods, chi-square test statistics, and p-values. Model components are:- additive polygenic genetic effects (G), sex/gender (S), coat colour (C), litter size (L) and residual environmental effects (E). Log likelihoods were calculated using multivar. Chi-square test statistics were obtained by taking twice the difference between the sub-model log likelihood and the full model log likelihood. In each case the degrees of freedom for the test was one. The p-value represents the area to the right of the chi-square test statistic value in the tail of the chi-square distribution with one degree of freedom

2.5. Discussion

The main finding from the familial aggregation analysis was that the variance of refractive error in this pedigree of Labrador Retrievers has a significant genetic component (additive genetic effects). That makes dogs unique; as the only known animal species with naturally occurring myopia, which is also inherited. It is the first time that this finding has been demonstrated in a non-human species. This makes the Labrador Retriever a more appropriate animal model of human myopia than current animal models where myopia is induced by form deprivation or lens induction. Other factors which make the Labrador an excellent animal model are the ability to test animals with non-invasive procedures free of sedation or anaesthesia, the rapid development of refractive error (compared to humans), large litter size with fast generation turnover, short gestation (60 days), similar ocular anatomy to humans, similar physiological response to drugs, and that multiple generations can be tested at any given time.

One major advantage of using canine pedigrees is the reliability of relationships, and the ability to engineer desired phenotypes/genotypes. In human studies, relationships within large pedigrees are ascertained through 'reported biological relationships' from members of families. Because familial aggregation analysis relies purely on phenotypic values with no known genotypic descriptors, there is no way of verifying the relationships between individuals. It has been reported that the rate of 'non-paternity' or 'non-mendelian inconsistencies' in human pedigrees varies from 0.8-30% (Bellis, Hughes et al. 2005), making statistical analysis of human pedigrees unreliable. It also makes any following genotyping very inaccurate, and requires careful data quality checks and analysis, which often leads to individuals being removed from genotyping analysis which is costly and reduces statistical power. However, with canine pedigrees, as identity of each dog is verified at the time of testing, these errors would be expected to be very small. Pedigree dogs are bred carefully with witnessed matings and often have interventions such as artificial insemination (AI) where sire identity is assured. Even where AI is undertaken with more than one sample (one litter with more than one sire), genetic identity of pups is verified after birth to allow registration. Bitches in heat are kept isolated to make sure 'accidental' matings do not occur. The ability to check a dog's identity has been strengthened by the utilisation of micro-chipping, which gives each animal a unique identifier that can be checked each time it is tested. Selected breedings and cross breedings, and back-crossing are also used to concentrate certain phenotypes in research breeding colonies. Back-crossing and other types

of breeding allow modes of transmission to be determined and mix desirable genotypes. Through engineered matings over several generations, modes of transmission can be determined.

Although the prevalence of myopia found in this pedigree cannot be generalised across the whole breed, an interesting feature about the prevalence and distribution of refractive error is that it closely resembles human studies, both in the prevalence of myopia (around 30%) and the degree of myopia which is most commonly found in human adolescents termed 'common' or 'school' myopia (Rose, Smith et al. 2001). The levels of myopia found in the canine pedigree were low in relation to similar studies of human families which often have myopia exceeding -15D. However, these higher levels of myopia; termed 'high myopia' seem to be a different form of the condition, in that myopia develops earlier in life, has greater risk of associated pathology, shows clearer genetic causation and occurs in a much smaller percentage of the population (Liang, Yen et al. 2004). High myopia has not been found in any reports of canine refractive error, in animals free of ocular pathology. It is 'common' myopia (low level, high prevalence, typically defined as <-6.00D) which is becoming increasingly prevalent in Western society, with high myopia increasing in prevalence to a lesser extent (Lin, Shih et al. 2004).

Our canine pedigree is also similar to human populations in that the variance of refractive error was explained through both genetic and environmental factors. Numerous studies have shown both the influences of genetics, through twin and family studies; as well as the effects of environmental influences such as education, near work and exercise (Rose, Morgan et al. 2002; Saw, Tong et al. 2004; Rose, Morgan et al. 2008). However, when dealing with children these factors are difficult to influence and control. The dog may represent a model where more of these factors can be monitored to study what most influences myopia development.

In the pedigree we studied, additive genetic effects accounted for about half of the variance in spherical equivalent refraction. This is a very similar level to that reported for human family studies (Biino, Palmas et al. 2005; Chen, Scurrah et al. 2007), although much less than the high levels reported for twin studies in which additive genetic effects account for over 80% of variance in refraction.

The fact that gender did not influence the level of refractive error is similar to that found in previous studies, although there have been somewhat conflicting results with some studies finding myopia was more prevalent in girls (Czepita, Zejmo et al. 2007).

Coat colour also did not show any association with refractive error. This variable was included in the analysis as it was another clearly defined phenotypic variable which could be defined for all animals. Coat colour in domestic dogs has been studied extensively at a genetic level as some coat colours are seen as more desirable and command higher puppy prices (Cadieu, Neff et al. 2009). The genes underlying the three coat colours in Labradors are controlled by at least nine alleles, and there is a commercially available test to test for these genotypes. In Labrador Retrievers, the genes underlying coat colour do not seem to affect iris pigmentation. However, in other breeds which have a different form of the merle gene (causing a variegated grey coat colour), there is an association with a merle allele and ocular anomalies and deafness (Strain, Clark et al. 2009). In future if other breeds of dogs known to carry a variant of the merle allele show naturally occurring myopia, coat colour may be an important variable to consider. Compared to previous studies of refractive error in dogs, this pedigree shows a much higher prevalence of myopia than previously reported, as well as a significantly more myopic average SER for the overall pedigree. Mutti et al initially tested a range of breeds in a study published in 1992 which demonstrated that naturally occurring myopia in Labrador Retrievers is a result of vitreous chamber elongation. Kubai et al (Kubai, Bentley et al. 2008) published a more recent study with a summary of all Labradors' tested in previous reports (Murphy, Mutti et al. 1997; Mutti, Zadnik et al. 1999) between 1991 and 2006, with an average SER of -0.12±1.19D (n=208) for Labrador Retrievers. This was a more hyperopic SER than found in our study of -0.41D, but a similar sample size and range of refractive error (our study (+1.65D to -5.38D) and that of Mutti et al. (+3.5D to -5.00D)). Both our pedigree and that of Kubai et al (Kubai, Bentley et al. 2008) found that entire litters of dogs tended to be myopic. Another difference found in previous studies of canine refractive error is that there have been no age criteria due to the population of dogs studied (vet hospitals) and animals were shown to get more myopic with age (Murphy, Zadnik et al. 1992; Kubai, Bentley et al. 2008). We have demonstrated in our population that age had no effect on refraction, but this was limited to dogs 1-8 years of age. It would be expected that an even greater degree of myopia would be found if we had tested dogs across all age groups due to lenticular changes.

One query this study has raised is the necessity for visual screening of dogs within service roles requiring the use of visual cues. The colony we have tested showed a significant prevalence of refractive error, most notably myopia. Currently, there is an emphasis on ophthalmological checks in guide dogs, with eye health being checked extensively over the first 18 months of life. Where dogs enter the breeding programme, they have ophthalmological checks every 12 months, to check for ocular pathology such as early cataract formation. There is currently no refractive screening programme in place for any service dog colony within New Zealand, and no reported screening takes place internationally. However, it could be argued given the current findings that refractive error checks are also important, as myopia seems to affect such a significant proportion of Labrador's within the Guide Dog colony. The difficult aspect to this type of screening would be what level of refractive error would necessitate withdrawal from the training programme. It has been previously shown that myopic defocus reduces the visual acuity of dogs (Murphy, Mutti et al. 1997). Previous studies have suggested that refractive error screening is advisable given the variation of refractive error, and clustering of myopia in families (Murphy, Mutti et al. 1997; Kubai, Bentley et al. 2008). New Zealand would be an ideal country to trial a refractive screening programme as guide dog services for the entire country are managed from one breeding and training facility, with regional staff managing local training for clients. There are also a relatively low number of guide dogs within New Zealand, so continued screening both on new puppies and existing guide dogs would be manageable.

Our study population differed from previous canine refractive error studies in that its members were a related sample originating from a common progenitor. It is probable that Labradors from different lines demonstrate a different intrinsic susceptibility to refractive error development. All previous large-scale studies of Labradors' refractive error have been from North American populations, whereas the New Zealand breed standard has different physical characteristics being based on the United Kingdom standard. Although it is difficult to quantify these differences as many of them are subjectively assessed, one area of difference between the standards is the ideal height at the withers (the highest point on the back of an upright dog, on the ridge between its shoulder blades). The US standard allows a greater variation and higher values for height (varying between 57.2-62.2cm for males and 54.6-59.7cm for females) than the UK standard (between 56-57cm for males and 55-56cm for females). Both standards agree on eye and coat colours, however in the US standard a

depigmentation of the eye rims is classified as a disqualification phenotype, which is not stipulated in the UK standard. Although these variations are minimal, if the underlying genetic variation which influences refractive error development was related to height variation this may be one cause for the differences observed in refractive error prevalence.

Because our population of Labradors originated from a service dog colony, their environment during development differs greatly from dogs living in a normal domestic environment. Service dogs live much like a human child, living inside a house full-time, with moderate time outdoors. They have an excellent diet and veterinary care. These factors may contribute to myopia development, particularly in the first six weeks of life when they live in a very small sheltered environment. These conditions may lead to higher levels of myopia development than if they had been raised outdoors which would occur if raised in the wild. However, another advantage of dogs is that they are free of environmental influences implicated in studies of children such as reading and computer work. Although, the amount of accommodation available to a dog is much lower than a child, even when young. Dogs are estimated to have 2-3 dioptres of accommodation which corresponds to a closes focus at approximately 1 metre, assuming that half of accommodation is exerted at maximum, in comparison a 10 year old child has at least 12D of accommodation which corresponds to a focal distance of 17cm.

The other main and somewhat unexpected finding from the familial aggregation analysis was that dogs from smaller litters are significantly (both statistically and clinically) more myopic than dogs from larger litters. There are many factors which would differ dependent on litter size. From a genetic perspective, it may be that the genetic loci for litter size is to some extent linked to that for refractive error development or eye growth. Litter size also affects birth weight, and influences competition for nutrition and physical activity. It would be expected that puppies born into larger litters would have more competition for food and therefore may have higher levels of physical activity. Physical activity has been shown to provide a protective effect against myopia in children and adolescents (Jacobsen, Jensen et al. 2008; Deere, Williams et al. 2009).

These factors required further investigation and analysis and are detailed in chapter four which discusses environmental factors including litter size, birth weight, growth rate and season of birth.

One limitation of this investigation was dealing with the high level of consanguinity in the pedigree. Although there are standard ways of defining these relationships, most software packages which deal with familial and segregation analysis are designed for human populations and cannot deal with inbreeding loops or multiple matings. Using the MORGAN package provided a way of looking at the pattern of phenotypes across the pedigree, but did not allow for mode of transmission analysis.

2.5.1. Future Directions

After the results outlined above were collated, the next appropriate step in this study was to take the relatedness of refractive error one step further and undertake genotyping to identify a loci for canine refractive error. Chapter three discusses the genetic portion of the study.

It would be interesting to repeat these methods on a pedigree of Labradors originating from different founders. Since this study is the first of its type in dogs, it seems likely that there are other pedigrees which would demonstrate higher levels of myopia than those found in this study. Because this study was conducted in New Zealand, and primarily in the North Island, there are relatively few Labrador Retriever breeders and most breeders share breeding animals, particularly sires, making the Labrador Retriever pedigree in New Zealand extensively inter-related. It would be interesting to sample pedigrees in countries with much larger numbers of breeders with greater genetic diversity.

To identify the need for service dog refractive error screening processes, it would be of interest to behaviourally test the effect of inducing/correcting refractive errors within dogs training to guide. As training guide dogs have set training courses and behavioural outcome measures, this would be relatively simple to implement with the aim of determining changes in behaviour coincident with the correction or induction of ametropia, and the level of ametropia where this behaviour becomes undesirable.

Chapter 3. Genetic Analysis

Introduction

Linkage and association studies comprise the two main methods of detecting the underlying genetic cause of disease, in particular those of complex traits with multiple loci (Elston 1998). Linkage studies are conducted in families which show segregation of disease phenotypes to look for shared genetic regions between affected individuals. Association studies are carried out in both related and unrelated groups, but tend to be conducted in populations with affected case groups and unaffected control groups.

This chapter will briefly explain the underlying methodologies of these types of studies, and then summarise the literature surrounding the current understanding of the genetic basis of myopia.

3.1.1. Linkage Studies

Linkage refers to the physical proximity of loci along a chromosome. Where two loci lie close together on a chromosome, they tend to be inherited together and co-segregate within families, in contradiction to Mendels law of independent assortment. Evidence of genetic linkage was first discovered by Morgan in 1911 using evidence from species such as Drosophila. Co-segregation can be broken up by recombination (the recombining of DNA during meiosis between paired chromosomes). The chance of recombination for two closely spaced loci reduces as the distance between the loci decreases (Leutenegger, Genin et al. 2002). Hence, recombination is dependent on the spacing between loci, although this is not a simple linear relationship. Linkage is defined by the parameter Θ which is the frequency of an odd number of recombination's between two loci. Even numbers of recombination's cannot be recognised because they look like parental types. For two loci which are far apart on a chromosome, Θ would be expected to be 0.5 as independent assortment occurs. Linkage analysis aims to find loci where recombination occurs with a frequency of less than 0.5. Recombinants occur with frequency Θ, and non-recombinants occur with frequency $1-\Theta$. The simplest estimate of Θ is the proportion of individuals within a sample that are

recombinants. However, this can be difficult in human populations because of the average small number of offspring in families, so methods have been developed to allow the combination of information between different families and populations.

Morton, one of the leading researchers and pioneers in the area of linkage analysis developed the LOD score method (logarithm of an odds ratio) (Morton 1955). The LOD score is based on an odds ratio which determines the probability of observing the specific genotypes in a family (given linkage) at a given recombination percentage versus the same probability computed conditional on independent assortment (Leutenegger, Genin et al. 2002). The result is that that high linkage values support the hypothesis of linkage at a given locus, and low values support no linkage in this area. The log of this value (the LOD score – see glossary) is taken so as to be able to easily combine information across studies by simply summing LOD scores (Leutenegger, Genin et al. 2002). A LOD score of 3 or greater is taken as statistically significant evidence of linkage; at this level the likelihood of linkage is 10^3 more likely than the likelihood of the loci not being linked (Rice, Saccone et al. 2001). A score of -2 or less is taken as definite evidence against linkage. A LOD score of 1.5 or greater is 'suggestive' of linkage.

The predicted frequency of alleles shared in related pairs, identical by descent (i.b.d), is calculated using the kinship coefficient. One way to decrease the sample size required is to select relative pairs who both suffer from a particular disease phenotype, and then compare the number of alleles i.b.d and calculate if this is greater than would be expected in pairs from a random sample (Genin and Clerget-Darpoux 1996).

Linkage analysis can be model based or model free (Huang and Vieland 2001). All such statistical analyses are based on a probability model, which is used to estimate parameters or test hypotheses about parameters. Model based analyses refer to the assumed mode of inheritance for the genetic loci of interest, for example dominant, recessive or sex linked. Model free does not assume any particular mode of transmission. Model based analysis is often referred to as 'LOD score' or parametric analysis, whereas non-model based analysis is referred to as non-parametric.

3.1.2. Association Studies

Association studies involve finding markers which are associated with a given disease phenotype. They involve studying disease traits at a population level rather than in related groups as in linkage analysis.

Linkage disequilibrium ('allelic association' or 'gametic phase disequilibrium') occurs when alleles are transmitted together more frequently than would be expected by chance and occurs when alleles are located closely on a chromosome (Elston 1998). Over time, recombination will eventually cause the alleles to be split and be inherited in equal proportions. In a randomly mating population, Hardy Weinberg equilibrium (see glossary) is a function of the recombination fraction Θ, and with each consecutive generation equilibrium becomes closer. This means that the closer the linkage, the longer it takes for the population to reach Hardy Weinberg equilibrium.

There are two main methods involved in association studies: the functional variant can be measured directly, or linkage disequilibrium between a marker and the functional variant can be measured. In the first case the genotype can be determined in a number of ways through DNA sequencing or immunological techniques. This gives a direct measurement of the genotype in relation to the phenotype. However, if random rather than functional markers are used, linkage disequilibrium is measured on a population level.

There are two forms of association studies depending on the origin of the subjects. Case-control studies generally involve unrelated individuals, recruited based on a certain phenotype, and matched unaffected controls. It is important in such studies to ensure that the cases and controls are matched well in terms of genetic origins, so they must be of similar ethnic background (Elston 2000). The underlying assumption in such studies is that a variant of a genetic marker, such as a microsatellite or Single Nucleotide Polymorphism (SNP), will be found distributed unequally between the two groups. One way of performing analysis in this type of study is to form a contingency table with expected and observed frequencies of genotypes. For example, for a single SNP there are six possible genotypes (AA, AB and BB) in cases and controls (Borecki and Suarez 2001). The contingency table can then be analysed directly using an observed-expected test statistic, which has a chi-squared distribution of two degrees of freedom (df). This statistic tests for departure from the expected values across cells in the table. The test statistic approximation is asymptotic, meaning that the analysis

becomes more accurate as data sets increase. The data can also be analysed with an assumed mode of transmission.

Control genotypes should show Hardy Weinberg equilibrium, provided that the underlying population shows random mating and is large enough in size. This means that genotypes AA, AB and BB should show frequencies of p^2, 2pq and q^2 where the frequency of allele A is p and allele B is q-1(Elston 1998).

The other type of association study involves related subjects, and has the advantage that rare disease alleles tend to concentrate in families. The transmission disequilibrium test (TDT) is a test which also studies alleles transmitted to affected offspring but takes into account both the linkage and disequilibrium underlying association. The TDT requires genetic information from both biological parents and offspring, and at least one parent must be heterozygous for the trait of interest for the test to be effective. TDT statistics are very robust against population stratification (Genin and Clerget-Darpoux 1996).

Haplotype analysis can also be used to detect genotypes where specific alleles across several SNPs cause disease states. The statistics described above analyse each SNP individually, but multiple testing is necessary in some situations. A Bonferroni correction to the p-values obtained from each SNP is overly conservative, since the SNP's may be in linkage disequilibrium and therefore results from each test are not independent. Other statistical methods including permutation testing and false discovery rate analysis models the number of expected false positive results and adjusts the p value accordingly making them less conservative than the Bonferroni correction.

The segregation of refractive error in families as been extensively discussed in chapter two, including a summary of studies which have analysed the heritability of ocular components which influence refractive error including corneal curvature, axial length, anterior chamber depth, lens thickness and astigmatism. Chapter two also discusses twin studies which have looked at the heritability of refractive error in MZ and DZ twin pairs, which presents convincing evidence that refractive error is strongly influenced by genetics as well as environment.

3.1.3. Current Candidate Loci for Myopia

Numerous loci for myopia have been mapped (see Table 3). It is of particular importance when studying a disease such as myopia to ensure that the population under investigation has

no other systemic signs of associated phenotypes as there are many syndromic disorders that have high myopia as a key feature. A current search of Online Mendelian Inheritance in Man (Online Mendelian Inheritance in Man(OMIM 2009)), a database listing the currently known candidate genes for human diseases produces 209 entries for myopia, with only 16 being specific 'myopia' genes (Table 3), the remainder being syndromes with myopia as a component.

Myopia Loci	Population (Def of myopia (D))	Loci (LOD score)	OMIM Reference	Reference
MYP1	Danish (<6D)	Xq28 (4.8)	310460	(Bartsocas and Kastrantas 1981; Haim, Fledelius et al. 1988; Schwartz, Haim et al. 1990)
MYP2	American (<-6D)	18p11.31 (9.59)	160700	(Young, Ronan et al. 1998; Lam, Tam et al. 2003)
MYP3	German/Italian (<-6)	12q21-q23 (3.85)	603221	(Young, Ronan et al. 1998; Farbrother, Kirov et al. 2004; Nurnberg, Jacobi et al. 2008)
MYP4	French/Algerian (High, <-6D)	7q36 (2.81)	608367	(Naiglin, Gazagne et al. 2002)
MYP5	English/Canadian (High, <-5.50D)	17q21-22 (3.17)	608474	(Paluru, Ronan et al. 2003)
MYP6	American (Ashkenazi Jewish Descent) (Mild-moderate <-1D)	22q12 (3.0)	608908	(Stambolian, Ibay et al. 2004; Stambolian, Ibay et al. 2006; Klein, Duggal et al. 2007)
MYP7	United Kingdom (twin pairs -12.12 - +7.25)	11p13 (6.1)	609256	(Hammond, Andrew et al. 2004)
MYP8	-	3q26 (3.7)	609257	(Hammond, Andrew et al. 2004)
MYP9	-	4q12 (3.3)	609258	(Hammond, Andrew et al. 2004)
MYP10	-	8p23 (4.1)	609259	(Hammond, Andrew et al. 2004; Stambolian, Ciner et al. 2005)
MYP11	Chinese (≤-5.00)	4q22-q27 (3.11)	609994	(Zhang, Guo et al. 2005)
MYP12	American (Northern European descent) (≤-6.00)	2q37.1 (4.75)	609995	(Paluru, Nallasamy et al. 2005; Chen, Stankovich et al. 2007)
MYP13	Han Chinese (≤-6.00)	Xq23-25 (2.75)	300613	(Zhang, Guo et al. 2006)
MYP14	American (Ashkenazi Jewish descent)(≤-1.00)	1p36 (9.5)	610310	(Wojciechowski, Moy et al. 2006)
MYP15	Hutterite (≤-6.00)	10q21.1 (3.22)	612717	(Nallasamy, Paluru et al. 2007)
MYP16	Chinese (≤-6.00)	5p15.33-p15.2 (4.81)	612554	(Lam, Tam et al. 2008)

Table 3 The current candidate loci identified for myopia defined by population, chromosomal location, OMIM identifier

Below is a summary of the 16 current myopia candidate regions:

MYP 1- Xq28

In 1981 Bartsocas and Kastrantas (Bartsocas and Kastrantas 1981) discovered a family consisting of 3 myopic brothers who had 5 myopic grandsons through their daughters (some of whom were mildly myopic). This presented a convincing model of X-linked myopia free of other systemic conditions. Haim et al (Haim, Fledelius et al. 1988) described such a syndrome in 1988 in a Danish family who presented with myopia, hypoplasia of the optic nerve heads and astigmatism. All affected males in this cohort also suffered from deutranopia. This condition was traced back over 5 generations to the island of Bornholm and was so named Bornholm eye disease. This family was then investigated by Schwartz et al (Schwartz, Haim et al. 1990) who, following linkage analysis, found a significant LOD score of 4.8 at F8C (θ=0). This finding positioned the gene at the distal region of the X chromosome in region Xq28 and this region was termed MYP1.

MYP2- 18p11.31

A genomewide scan was conducted by Young et al (Young, Ronan et al. 1998) on 8 multigenerational families with an autosomal dominant pattern of high myopia of ≤-6.00D. The mean age of myopia onset in these families was 6.8 years. The average refractive error of affected individuals was -9.48D with a range of -6.00D to -21.00D. Following a genomewide scan, a highly significant LOD score of 9.54 was found at a locus on chromosome 18p, at marker D1S6481 with a recombination fraction of 0.0010. Haplotype analysis further refined this locus to a 7.6-cM interval between markers D18S59 and D18S1138 (centromerically) on 18p11.31 through recombination events.

MYP3- 12q21-q23

Young et al (Young, Ronan et al. 1998) found a second locus for high myopia at chromosome 12q21-23 in a large family of German/Italian descent. Inclusion criteria were myopia onset at less than 12 years of age, myopia of ≤-6.00 and affected status in at least two generations. The average spherical component of myopia was -9.47 dioptres, and the average age of first diagnosis was 5.9 years. The maximum LOD score with 2-point linkage analysis was 3.85 at a recombination fraction of 0.0010 for markers D12S1706 and D12S327. A 30.1cM interval on 12q21-23 was defined at this locus by recombination events. Young et al identified two possible candidate genes in this region, lumican (12q21.3-q22) and decorin (12q23).

Nurnburg et al (Nurnberg, Jacobi et al. 2008) went on to isolate a candidate region on chromosome 12q14.3 to 12q21.31 using a 10K whole genome SNP array in an extended family (28 members) of German descent. The inclusion criterion for affected members of the family was myopia of ≤-5.00D, and was studied over a six generation pedigree. Combining the region of the original MYP3 locus defined by Young et al (Young, Ronan et al. 1998) and the findings from the whole genome chip, the region was able to be narrowed significantly to between microsatellite marker D12S1684 and SNP marker rs717996. This reduced the region of the original MYP3 interval from 30.1 to 6.8cM, a region consisting of 25 possible genes.

MYP4- 7q36

Naiglin et al (Naiglin, Gazagne et al. 2002) identified a novel locus for autosomal dominant high-grade myopia (≤ 6D) in 21 French and 2 Algerian families. Previously identified myopia loci were excluded, and multipoint analysis showed suggestive evidence of linkage to chromosome 7q36, with a maximum multipoint LOD score of 2.81. No locus heterogeneity was detected. An 11.7cM critical region extending from D7S798 to the telomeric end of the chromosome was defined using a recombination event between markers D7S798 and D7S2546.

A later study failed to replicate the significant association at 7q36 but did find a significant interval at chromosome 7p15, of 7.81cM (Paget, Julia et al. 2008).

MYP5- 17q21-q22

In 2003 Paluru et al (Paluru, Ronan et al. 2003) identified a novel locus for high myopia in a family of English/Canadian descent. The criteria were myopia onset before 12 years of age, no other systemic or ocular manifestations and at least two generations affected. Myopia was classified as ≤-5.00. 22 family members were studied. Three previously identified AD high myopia loci were studied: 18p11.31 (MYP2), 12q22-q23 (MYP3) and 7q36 (MYP4). All previous candidate loci were excluded in this family. Marker D17S1290 showed a LOD score of 1.94 suggestive of linkage, so fine mapping was undertaken of 17q with additional markers. A LOD score of 3.17 was identified at marker D17S1604. This narrowed down the critical region to a 7.71cM area between markers D17S787 and D17S1811 on the long arm of chromosome 17.

MYP6- 22q12

Most studies of myopia genetics have focused on high myopia (≤-5.00D). In 2004 Stambolian et al (Stambolian, Ibay et al. 2004) sought to identify a myopia susceptibility gene related to mild/moderate myopia, which is a very common disorder particularly in developed populations. Stambolian initially studied an Ashkenazi Jewish population as they have been shown to a have a higher prevalence rate of myopia than other white American or European populations. A family of one known ethnicity also offers the advantage of reducing heterogeneity between subjects. Forty-four large American families of Ashkenazi Jewish descent were studied, with at least 2 affected siblings in each family. Individuals with refractions of at least ≤-1.00D or less in each meridian of both eyes were classified as myopic. Subjects were chosen to fit a model of dominant inheritance. A significant maximum multipoint parametric heterogeneity LOD score of 3.54 was observed at marker D22S685. The parametric multipoint HLOD scores exceeded 3.0 for a 4cM interval. Results from linkage analysis identified a new locus on chromosome 22q, with some evidence for a second locus on chromosome 14q. The area on chromosome 22q was also significant because there are a number of genes in this region which are known to be strongly expressed in the eye including heme oxygenase 1, RNA binding protein 9, and minichrom maintenance deficient 5 (Stambolian, Ibay et al. 2004).

MYP7- 11p13

Hammond et al (Hammond, Andrew et al. 2004) undertook a classical twin study to establish the heritability of refractive error. A total of 506 twin pairs were measured for refractive error, 280 DZ and 226 MZ twin pairs. The mean spherical equivalent of the 506 twin pairs was +0.39D, with a range of -12.12D to +7.25D. Only 7 of the twin pairs fell into the category of high myopia (≤ -6.00D). The heritability of refractive error was found to be 0.89, which is similar to that found in previous studies (Hammond, Snieder et al. 2001; Lee, Klein et al. 2001; Lyhne, Sjolie et al. 2001). A genome wide linkage scan was then completed on 221 DZ twin pairs to search for potential susceptibility loci for myopia. Myopia was analysed as a continuous trait. The linkage scan found four potential loci with significant LOD scores. The highest LOD score (6.1) was found at chromosome 11p13 (MYP7). The other peaks occurred at 3q26 (MYP8), 4q12 (MYP9) and 8p23 (MYP10). The LOD scores at these loci were 3.7, 3.3 and 4.1 respectively. At the 11p13 locus, Pax-6 was the most likely candidate gene as its location fell directly under the maximum peak and is known to have several functions associated with eye growth and development. A fine mapping study of this

region was completed with flanking markers. However, no phenotypic association was found at the Pax-6 locus.

Han et al investigated the pax6 locus in a group of families of Han Chinese origin with high myopia (Han, Leung et al. 2009). Tagging SNP markers and other SNP markers within the Pax-6 region were chosen. The findings were that SNP polymorphisms in the 3' region of the Pax-6 gene may increase susceptibility for developing high myopia.

MYP8- 3q26

As described above, Hammond et al (Hammond, Andrew et al. 2004) found four significant loci in twin pairs in a linkage study of twin pairs. MYP8 is the region at 3q26 that was found to have a LOD score of 3.7. No candidate genes have been studied in this region.

MYP9- 4q12

MYP9 was also discovered by Hammond et al (Hammond, Andrew et al. 2004) in their large classical twin study. MYP9 was found at locus 4q12 with a LOD score of 3.3. To date no candidate genes have been studied within this region.

MYP10- 8p23

MYP 10 was the final region implicated in Hammond et al's 2004 twin study (Hammond, Andrew et al. 2004). This region was found to be significant at 8p23 with a LOD score of 4.1. No candidate genes have yet been further studied in this region.

MYP11- 4q22-q27

Zhang et al (Zhang, Guo et al. 2005) studied a Chinese family of Han descent with 12 individuals affected with high myopia in a pattern suggestive of autosomal dominant inheritance over four generations. Myopia was defined as $\leq$ -5.00D using cycloplegic retinoscopy and all affected individuals had developed myopia before school age. Markers surrounding the four known loci for AD high myopia revealed no statistically significant evidence of linkage. Linkage analysis revealed a novel locus on chromosome 4 with a maximum LOD score of 3.11, encompassing a region of 20.4cM between markers D4S1578 and D4S1612. A candidate gene, RRH (retinal pigment epithelium derived rhodopsin homolog) was studied, but sequence analysis revealed no causative mutations.

MYP12- 2q37.1

Paluru et al (Paluru, Nallasamy et al. 2005) studied a large family of Northern European-American descent, 31 members were studied with 14 affected. The average refractive error of the affected individuals was -14.46D. A novel region was found for AD high myopia using a genomewide linkage study. The novel locus on chromosome 2q37.1 had a maximum multipoint LOD score of 4.75, at marker D2S2344. Eleven candidate loci that were already known to be implicated in high myopia were screened but were not significant. Fine mapping was conducted on possible candidate genes within this region, S-Antigen and diacylglycerol kinase-delta (DGKD) were sequenced and no causative SNP's were found.

MYP13- Xq23-q25

A study by Zhang et al (Zhang, Guo et al. 2006) studied X-linked high myopia in a family of Han Chinese ethnicity. The family had six affected individuals over four generations with myopia ranging from -6 to -20 dioptres. A linkage study of the X chromosome found a locus differing from the previously described MYP1 region. Myopia was analysed as an X-linked recessive trait with full penetrance and an allele disease frequency of 0.0001. Three of the six individuals had a concomitant colour vision defect. Reduced cone response on ERG, reduced stationary visual acuity, a temporal crescent at the optic disc and thinning of the retinal pigment epithelium suggested a level of cone dysfunction. Linkage analysis ruled out the previous MYP1 region but found 2-point LOD scores of 2.75 and 2.29 at markers DXS1001 and DXS8059. A conserved haplotype was also identified between DXS1059 and DXS8059. This haplotype was present in all affected individuals and unaffected carriers but not in unaffected males. Recombination events set the telomeric boundaries in a region of 25cM (14.9Mb) between DXS1210 and DXS8057 at Xq23-q25. There are approximately 101 genes in this region, of which GUCY2F, GLUD2, GRIA3, BIRC4, STAG2 and LRCH2 were possible candidate genes.

MYP14- 1p36

Wojciechowski et al (Wojciechowski, Moy et al. 2006) investigated the same Ashkenazi Jewish families previously studied by Stambolian et al (Stambolian, Ibay et al. 2004) which resulted in the discovery of the MYP6 loci. A total of 411 individuals were studied from 49 families with 2.7 generations each on average. Of the participants, 86.9% were myopic ($\leq$-1.00D). A genome-wide scan was completed using 387 microsatellite markers. A maximum LOD score of 9.54 (p=0.065) was found between markers D1S552 and D1S1622

on a 49.1cM region on chromosome one for REF (refraction), and a maximum LOD score of 8.74 (p=<0.005) for the same region for LTR (log-transformed refraction).

MYP15- 10q21.1

Nallasamy et al (Nallasamy, Paluru et al. 2007) conducted a study on a family of Hutterite descent in South Dakota conducting a genome wide scan using microsatellite markers on 29 family members, 7 of whom were myopic, with the average refractive error being -7.04D. Genetic regions previously implicated in syndromic or non-syndromic high myopia were excluded. All regions giving positive LOD scores were finely mapped giving a significant LOD score of 3.22 at marker D10S164. Fine mapping and haplotype analysis identified a 2.67cM region on chromosome 10q21.1. Haplotype analysis identified two distinct haplotypes segregating with high grade myopia suggesting two distinct mutations on the same gene.

MYP16- 5p15.33-p15.2

Using a genomewide scan and linkage analysis, Lam et al. (Lam, Tam et al. 2008) investigated the genetic component of 3 Chinese pedigrees from Hong Kong with autosomal dominant high myopia. They found a maximum 2-point lod score of 4.81 at D5S2505 at θ= 0.00. Haplotype analysis narrowed the linkage region to 5p15.33-p15.2 with a 17.45cM interval.

Other Identified Regions

A large linkage study for high myopia has recently been completed in a group of 254 families, gathered from five different research groups (Li, Guggenheim et al. 2009). The study replicated myopia regions MYP1, MYP3, MYP11, MYP12 and MYP14 as well as identifying a novel region at chromosome 9q34.11.

Candidate Genes for Myopia

Whilst 16 candidate regions have been identified in a variety of populations, these regions consist of thousands of possible genes. Within these regions genes which are known to regulate ocular growth or be associated with visual physiology have been isolated and studied using fine mapping. Most of these studies have analysed individual genes, rather than attempting to identify haplotypes involved in myopia development. As with myopia

candidate regions, many of these genes have shown significant results in some populations, but have failed to replicate the same results in other groups.

Pax-6, a gene which is know to regulate eye development showed several variants associated with high myopia susceptibility in a population of Han Chinese (Han, Leung et al. 2009). However, further analysis in a different population found no association with Pax-6 or SOX2, another gene which regulates eye development (Simpson, Hysi et al. 2007). The myocillin myopia susceptibility gene (MYOC) has also been identified in a Chinese cohort with high myopia (Tang, Yip et al. 2007). This association with MYOC was also reported in a smaller Croatian cohort with high myopia (Vatavuk, Skunca Herman et al. 2009). The gene, UMODL1, was identified in a Japanese population affected with high myopia, finding one significant SNP on chromosome 21q22.3 following the region being identified in a genome scan (Nishizaki, Ota et al. 2009).

Collagen related genes have long been suggested as possible candidate genes for myopia susceptibility. To date an association has been found between 2 SNP's on the collagen 2 alpha 1 gene (COL2A1) and myopia (Mutti, Cooper et al. 2007). There are also reported associations between polymorphic changes on collagen genes and Stickler syndrome characterised by high myopia (Olavarrieta, Morales-Angulo et al. 2008).

Other genes have been implicated but have shown contradictory results between studies. The collagen type 1 alpha 1 gene (COL1A1) showed an association with high myopia in a Japanese cohort (Inamori, Ota et al. 2007) but not in a later study (Nakanishi, Yamada et al. 2009). The transforming growth factor-β-induced factor gene (TGIF) (Pertile, Schache et al. 2008), membrane-related frizzled-related protein (MFRP) gene (Metlapally, Li et al. 2008), Lumican gene, TGF-β1 and hepatocyte growth factor genes have also shown no associations with high myopia (Wang, Li et al. 2009).

3.1.4. Consanguinity and Inbreeding

Studying populations with limited genetic diversity, such as those that are geographically isolated has advantages, particularly when studying complex traits (Arcos-Burgos and Muenke 2002). Studying these types of human populations including the old order Amish and Finnish populations has resulted in the discovery of several genes in conditions arising from recessive transmission. These types of communities offer many advantages for the study of disease using genome wide mapping strategies. Most of these benefits are a result of the

founder effect (see glossary), the loss of genetic variation due to the establishment of a population from a small number of founding individuals. These advantages include the high level of inbreeding, which has resulted in an increased prevalence of recessive conditions. Well structured multi-generational pedigrees provide historical information about the development of rare conditions which usually occur across families who also share common environments. These conditions allow the study of populations using case-control designs, or transmission disequilibrium tests which both study linkage disequilibrium in populations. Haplotype sharing between affected individuals can then be completed to further analyse critical regions of DNA. One limiting factor that inbreeding introduces to genetic studies is the effective reduction of population size. Increasing the level of genetic homozygosity, one of the key findings in inbred populations, also reduces the number of independent genomes, creating a new effective population size (Nordborg 2000).

3.1.5. SNP Arrays

Single Nucleotide Polymorphisms (SNP's) are a genetic variant at a single base site within the DNA code. They are the most commonly found DNA variant in genomes, with approximately 10 million SNP's having been detected in the human genome (Kruglyak and Nickerson 2001). SNP's are ideal markers for studies of genetic disease due to their abundance, and stability both through evolution and within populations. Because an individual inherits one copy of each SNP position from each parent, the genotype at each position is often labelled as AA, AB, or BB.

A Single Nucleotide Polymorphism array is a tool used in molecular biology to detect genetic polymorphisms across a genome. The basic principles of SNP arrays are the same as DNA microarrays combining the technologies of DNA hybridisation, fluorescence microscopy and solid surface DNA capture. While the first commercially available human SNP array had only 10,000 SNP's (Wang, Fan et al. 1998), current arrays can accurately (>99%) genotype over one million SNP's across the human genome in each individual (LaFramboise 2009). Every SNP site on an array is matched to a range of probes which are each 25 nucleotides in length. Each probe is complementary to the DNA sequence around the SNP site. A range of probes are used to either match perfectly to the DNA sequence surrounding the SNP (a perfect match probe, PM) or for the central base to mismatch (a mismatch probe, MM). There is a pair of probes which corresponds to either Allele A or B, the results from the four

probes (PM_A, MM_A, PM_B, MM_B) (in the form of fluorescence intensity) are then combined to infer a genotype AA, AB or BB (LaFramboise 2009).

3.2. Aims of Chapter Three

3.2.1. Aim 3: To design a genetic association study with the potential to identify candidate regions for canine refractive error

Once Aim 2 established that refractive error in this pedigree displayed a significant genetic component, genotyping analysis was the next appropriate step in attempting to identify a possible genetic locus/loci for canine refractive error.

There were several preliminary steps necessary to develop methodology within this aim including establishing the best DNA sample collection method in dogs and verifying the best techniques for verifying relatedness between dogs.

There are several approaches that could have been used to address this aim, but it was decided to design a pilot study with a view to using the recently developed canine SNP array, as this gives genome-wide coverage. The canine SNP array was recently developed by the Broad Institute (Karlsson, Baranowska et al. 2007). The sampling strategy was to use a case-control design with 50 myopic dogs from the pedigree constructed in Aim 1, and a control group of non-myopic, unrelated Labrador Retrievers from other breeding colonies around New Zealand.

3.3. Methods

3.3.1. Contact with Breeders

Following the finding that there was significant genetic component to the refractive error found in the GDS population (discussed within chapter two), a second group of unrelated dogs was recruited in preparation for genetic analysis. A database of breeders was constructed from breeders associated with the New Zealand Kennel Club (NZKC) and various Labrador Retriever Clubs around New Zealand. Letters were sent to the three main clubs around New Zealand: the Auckland Labrador Retriever Club, the Wellington District Labrador Retriever Club and the Canterbury and Otago Labrador Retriever Club. Letters were also sent to all individual breeders around the Auckland and Waikato districts. The aim of contacting breeders was to test the breeding animals at each facility rather than to ascertain the names of owners. It was important to keep the samples as unrelated as possible, both between the case and control populations and also within the control population itself. Therefore breeding dogs represented an ideal population as they tend to be a group with ideal physical characteristics, but also tend to be minimally related to reduce the level of inbreeding. Also, breeders often have a group of imported animals, to increase the gene pool, which decreases the relatedness within the kennel.

Refractive error measurements were taken as previously described in chapter two. Validity of breed, age and pedigree information was ascertained using New Zealand Kennel Club records (www.nzkc.org.nz).

3.3.2. DNA Sample Types

The initial aim was to determine the best method of collecting canine DNA in terms of ease of collection, invasiveness, portability, DNA yield and DNA quality. The gold standard for human DNA samples is from whole blood. However in dogs sample collection involves collection by a veterinarian, and taking blood from the jugular vein in the neck, creating discomfort and anxiety for the animal. It also involves clipping the hair around the neck, which was not acceptable to owners of show dogs. Blood collection is also less convenient for dog owners and expensive. Several studies had investigated the efficacy of other types of DNA samples in dogs including buccal swabs, saliva and nail samples (Oberbauer, Grossman et al. 2003; Short, Kennedy et al. 2005; Karlsson, Baranowska et al. 2007). Initially we

compared the ease of collection between buccal swabs and toenail samples, and the ease of subsequent DNA extraction and amplification.

A small pilot study was conducted to analyse various sample types. These samples included blood, buccal swabs and toe nails. Blood is undoubtedly the standard sample type in human studies due to DNA yield and quality, however there has been a move recently to using buccal and saliva samples (including buccal swabs, mouthwash kits and saliva collection kits) for human studies due to the ease of collection and the ability to post samples (Borthakur, Butryee et al. 2008). We obtained a blood sample from one dog, collected toenail samples from two dogs and took buccal swabs with standard cytology brushes from two dogs. Protocols previously reported were used to extract the various sample types (Oberbauer, Grossman et al. 2003). To assess the success of amplification, two primers were designed for two canine genes. Each sample was then amplified according to the manufacturers recommendations in terms of cycle number and temperature. Each sample type (buccal, blood, toenail) was extracted on 3 separate occasions.

During the early phases of this study, saliva collection kits were becoming popular in human studies. No animal kits were available, but using a kit designed for saliva collection in infants, a large proportion of the examined dog population had DNA samples collected with Oragene (DNA Genotek, Ontario) saliva kits (as well as buccal swabs). Oragene kits have been demonstrated to provide higher yields and better quality DNA than either buccal swabs or mouthwash kits in human studies (Rogers, Cole et al. 2007).

3.3.3. Canine DNA Sample Collection Procedure

3.3.3.1. Buccal Swabs

DNA samples were collected by two methods depending on the ease of collection. Most dogs had buccal swabs collected, with between 1-4 swabs collected per dog from various locations around the mouth. Brushes were rubbed along the gums and cheeks of both sides of the mouth for at least 30 seconds. Masteramp remote site testing brushes were used (brushes in cases), which allowed swabs to be posted when necessary. Swabs were stored at -20°C until extraction. Swabs were labelled at the time of collection with the name of the animal, the swab number and the date of collection. A diagram was annotated with the location of each swab collected.

Prior to collection, dogs were kept in an area free of water, other animals, food and toys to prevent contamination.

3.3.3.2. Oragene

At the beginning of this study the use of Oragene kits and saliva as a type of DNA sample was beginning to increase in popularity for human studies (Rylander-Rudqvist, Hakansson et al. 2006) due to the ease of collection and storage. However, the use of Oragene for animal studies involving genetic analysis was in the early stages. As the collection and extraction protocols and kits were in development, several types of collection vials and swabs were trialled during the project. All of these kits used the same amount of Oragene DNA stabilising solution (2ml). The different kits and collection sponges used are shown in Figure 8.

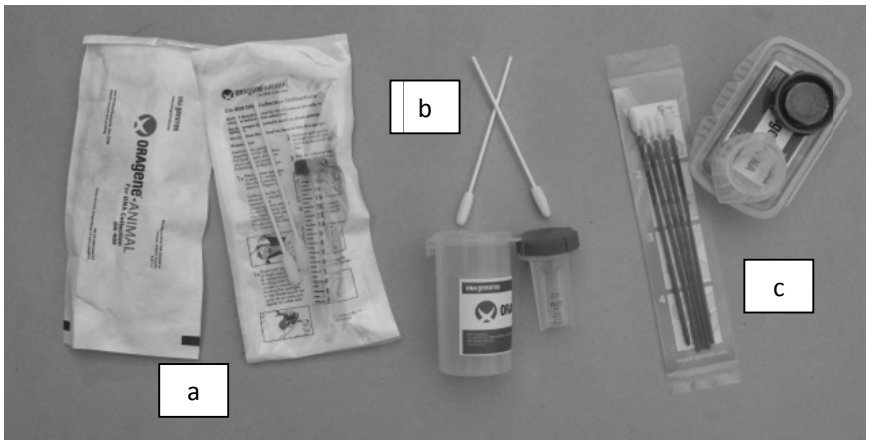


Figure 8 The various Oragene kits used. (a) The recently released final version the oragene animal kit (b) the beta version animal testing kit with larger sponges. (c) The human infant kit which was initially used with smaller sponge tips

A group of samples were collected using Oragene kits (animal testing version) (in addition to buccal swabs). There were two methods of collecting saliva using these kits (or a combination). Saliva was either collected directly into the vial (required sample volume of 2ml) (Figure 9 (a)), or collected using sponges which were placed into the mouth to absorb saliva. When collected using sponges, there were two methods to collect the sample, the sponges could either be moved around the mouth to absorb saliva in different regions (Figure 9 (b)) or could be placed inside the lower lip and held still (Figure 9 (c)) for at least 30 seconds. Oragene kits had the advantage that once the DNA stabilising solution had come into contact with the saliva sample it was stable at room temperature for months to years. This meant that the kits were ideal for posting to dog owners for home collection. Initially

the kits supplied were composed of a collection vial (with DNA suspension solution) and five small collection sponges. Following initial collections, a larger version of the sponges were provided, where only two sponges were used rather than five.

Figure 9 Various ways of collecting saliva using the oragene kit. (a) saliva collected directly into the vial (b) the sponges moved around the mouth to absorb saliva (c) the sponges held still inside the lower lip. For methods (b) and (c) between 3 and 5 sponges were collected.

3.3.4. DNA extraction and Preparation

3.3.4.1. Buccal Swabs

DNA was extracted using a Roche High Pure PCR Template Preparation Kit (Roche Diagnostics) with the protocol for suspended cell preparations. A modification was added before the protocol to allow extraction of cellular material into a solution. Two brushes were placed into 700µl of milliQ H^20 in a 1.5ml Eppendorf tube and heated at 40°C for 10 minutes with vigorous twirling and rubbing of the brushes within the solution. The brushes were then removed from the solution and discarded. The tubes were then centrifuged for 1 min at 13,000 rpm. 500µl of the solution was removed and retained and the Roche protocol was then continued for the remaining 200µl. At the last step the DNA was stored in a buffer volume (TRIS HCl pH 8.0) of 100µl rather than 200µl in an effort to increase DNA concentration (DNA concentration was required to be 50ng/µl for SNP genotyping). Any remaining brushes remained in storage at -20°C.

3.3.4.2. Oragene Samples

Oragene samples were stored at room temperature until extraction. Samples were processed following the standard protocol (DNAgenotek 2008). Where saliva was collected into sponges, the DNA was collected by the adapted protocol from DNAGenotek (dnagenotek), and DNA was stored in Roche Elution Buffer (Roche Diagnostics). As samples were

unequal a 500µl aliquot was removed and the remainder of the sample stored at -20°C in the 50ml conical centrifuge tube (sponges) or original collection vial (saliva).

3.3.4.3. DNA Quantification

Yield and quality of DNA was checked with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Delaware, USA) with 2 µl of sample. Yield (ng/ul) and 260/280 and 260/230 ratios were calculated. Blank measurements were calculated using the Roche Elution buffer (10 mM Tris-HCl pH 8.5) which was used to dilute DNA from both the buccal swabs and Oragene kits.

3.3.4.4. DNA Concentration/Dilution

As a DNA concentration of 50ng/µl was required for the following SNP array processing, concentration or dilution of samples was necessary following DNA quantification. A Speedivac DNA concentrator (Thermo Scientific, Delaware, USA) was used to concentrate samples. From the initial sample volume of 100µl, the volume was reduced to 20µl and DNA quantification was repeated until in the correct range (45-55ng/µl). Where concentration was too high, the samples were diluted with Roche elution buffer (Roche Diagnostics). Where DNA yield was too low to allow a sample volume of 5µl with 50ng/µl a new sample was attained or an alternative sample used.

3.3.5. Ascertainment of relationship

There are several measures of relationship within large pedigrees. Because of the extensive inter-relatedness within this pedigree, inbreeding and kinship coefficients (see glossary) were calculated for each animal, and each possible pair of animals within the case/control groups.

Prior to measurements of inbreeding and kinship coefficients, a pedigree was constructed containing all Labradors tested including those within the GDS colony and those from outside breeders. It was important to measure the relationships of all dogs within New Zealand because many of these dogs were descended from common ancestors. The database included Labradors from major kennels (breeding dogs only) within New Zealand and was exported from Breedmate Pedigree Explorer into Microsoft Excel (Microsoft Corporation, Chicago) and made into a text file. A total of 1187 dogs were included in this pedigree. All individuals were numbered between 1 and 1187. A computational limitation of the program was that both parents had to be included, so where only one parent was known, both parents

were entered as unknown. As many generations as possible were entered, with a maximum of 12 generations. The initial pedigree was processed through pedcheck software, which is part of the MORGAN package (described in chapter 2), which is used to detect errors in the pedigree such as gender misclassification and self-parenting. Pedcheck calculated pedigree statistics such as number of individual nuclear families, number of males and females and number of founding individuals. The output pedigree was then re-formatted and run through the KinInbcoef program which is part of the CC-QLS software (Karigl 1981; Thornton and McPeek 2007). The output from KinInbcoef provided files in the correct format for future genetic association analysis. As the association analysis was inevitably going to include dogs with a level of relatedness, the CC-QLS software package was selected that could include these relationships during genotyping analysis (Bourgain, Hoffjan et al. 2003). KinInbcoef also calculated kinship coefficients for specified pairs of individuals, as well as inbreeding coefficients for individuals. Kinship coefficients were calculated for all possible combinations of individuals for the case and control groups being studied in the genetic portion of the study.

3.3.6. Subject Selection for SNP Array Study

A case-control association study design was used with the aim of maximising the statistical power in a small sample size. The group of cases (50 myopic dogs) was gathered from the initial population tested in the segregation analysis. The most myopic dogs were used where DNA was available and none of the exclusion criteria applied. Some of the dogs included in the segregation analysis were lost to follow-up so DNA samples were not available. The control group was chosen from a separate population of Labrador Retrievers from breeders around New Zealand. It was assumed that the case and control groups were essentially unrelated even though there were some distant shared ancestors due to the nature of the breeding community in New Zealand.

Although there are some advantages to having related cases and controls in that the two groups come from the same population (Visscher, Andrew et al. 2008) (removing problems associated with population stratification), it also is disadvantageous in that it reduces the power to detect association due to the inflation of control frequencies towards case frequencies. Using related individuals also reduces the amount of information compared to using the same number of unrelated individuals due to shared alleles.

As the canine SNP arrays are still in the development phase and are not widely used there was a constraint on manufacturing of the SNP arrays, only being manufactured in runs of 90. However, this was similar to the initial estimated sample size of 100 (50 vs. 50 cases and controls) required for a pilot study to validate the efficacy of running a genome wide study of canine refractive error.

The sample size of 90 was chosen due to the complex nature of the trait, the pedigree not showing a clear recessive or dominant mode of transmission. If a trait was clearly dominant, an initial run of approximately 30 cases and controls would suffice for a pilot study (Karlsson, Baranowska et al. 2007). Due to the recent development of the canine SNP array it has not been widely used for canine disease traits, but in the majority of studies initial sample sizes have been less than 100 dogs per breed (Awano, Johnson et al. 2009; Wood, Ke et al. 2009). Due to the canine SNP arrays having a selected group of markers, additional individuals can be studied at a later time and combined to increase the sample size.

3.3.7. SNP Array Design

SNP arrays are able to analyse several different parameters of the genome. Current human SNP arrays have over one million markers of genetic variation located on each array. These arrays measure SNP polymorphisms and also allow measurements of copy number variation and loss of heterozygosity.

As the canine genome and subsequent SNP arrays are much later designs and less regularly investigated than equivalent human technology, the canine array has a comparatively lower number of genetic markers on the latest array, with one hundred thousand SNP's. The design of the 500K human SNP array and the canine version 2 (v2) array are similar and follow the same protocols, but there are also differences. Each gene on the 500K human chip is represented on the array by 10 probe pairs (per allele), with each probe made up of a 25 base oligonucleotide characteristic of the gene. Each probe pair is made up of one perfect match (PM) probe that should hybridise as the sequence matches, and one mismatch probe (MM) which should not hybridise as the central base is inverted, acting as an internal control. However, in the canine design, only PM probes are used.

The version 2 canine array is a PM probe only (20 probes/SNP) WGSA (whole genome sampling array) design, which can detect a total of 127, 000 SNPs. These SNPs were chosen from the 2.5 million SNP map generated as part of the dog genome project. A "v2 platinum"

set of 49,663 SNPs was selected to include accurate and robust SNPs, using a panel of >10 diverse breeds. SNP's were chosen on the basis of technical performance and location, with markers evenly distributed across the genome, and with multiple breed representation. All markers proved to be polymorphic in a range of dog and wolf breeds, although the amount of polymorphism varied depending on the breed (Karlsson, Baranowska et al. 2007).

3.3.8. Stages of Processing.

The processing of Affymetrix SNP arrays are completed at a commercial facility (Affymetrix centre, centre for Genomics and Proteonomics, University of Auckland), so only a brief description of the process is described below.

Genomic DNA was prepared to be at a concentration of 50ng/µl in a TRIS/reduced EDTA buffer. DNA was required to be of high quality, double stranded, free of PCR inhibitors and not be degraded (STY sites must be intact at both ends of strand to allow adaptor ligation). 5µl was used for each sample, giving total DNA of 250ng for each array. It was important that Genomic DNA was free of DNA from human or other organisms, as restriction enzyme amplification was not canine specific (contaminated or mixed DNA can show up as high detection rates and low call rates). The second step is restricted enzyme digestion. The protocol for the canine SNP array is the same as the human 500K mapping assay, except that only one of the enzymes is used. In the human array, both STY and NSP restriction enzymes are used. In the canine array just the STY enzyme is processed. Ligation is then completed with a common primer sequence added to digested fragments so that a universal primer can be used for polymerase chain reaction (PCR). The PCR step preferentially amplifies restriction fragments that are sized between 200 and 1000bp. The sequence complexity of the PCR products is estimated to be approx 60 Mbases, which represents a 50-fold reduction in sequence complexity. The one primer used in PCR is the forward strand of the adaptor; thus only two oligonucleotides are necessary for genotyping over 100,000 SNPs. Following PCR purification, the standard protocols are used to complete fragmentation, labelling and hybridisation. There are several QC checkpoints throughout the protocol; amplified DNA is viewed in a gel after PCR and fragmentation stages.

Following hybridisation, fluorescence patterns across arrays are analysed to predict alleles at each SNP site.

3.4. Results

3.4.1. Refractive Error Data – Case and control groups

3.4.1.1. Case group

As discussed in chapter two, 116 dogs were included in the familial aggregation analysis to determine the pattern of refractive error in the large pedigree from GDS. After analysis, dogs from this colony continued to be tested for refractive error, particularly those from younger generations who did not meet age criteria prior to the analysis. A further 64 dogs were tested using cycloplegic retinoscopy (between June 2007-December 2008), giving a total of 180 dogs tested from this colony.

The refractive error distribution is shown in Figure 10. Of the total group of GDS Labradors tested, the average refractive error was -0.47D (SER ± 1.38D). The average SER for the RE was -0.48±1.39D and for the LE was -0.49±1.33D. The average age at the time of testing was 3.91±2.6 years. The average difference in SER between the right and left eye was -0.01058. Emmetropia was present in 55% of the GDS sample, 37% were myopic and 8% were hyperopic. The range of refractive error in this family was +1.65D to -5.37D. The level of astigmatism was generally low in the population. Clinically significant astigmatism (>0.50DC) was present in 15 eyes (4%). The range of astigmatism was -0.50 to -2.60DC. Nine animals had anisometropia of more than one dioptre (range 1-4.49D). One dog was antimetropic.

3.4.1.2. Control Group

Dogs from other NZ breeders were tested at several dog shows throughout the North Island. Nine dogs were tested at the Auckland Labrador Retriever club ribbon parade (Ardmore, Auckland), 17 dogs were tested at the Manawatu Kennel club show (Palmerston North) and a further 20 dogs were tested at 3 other breeders in the greater Auckland region. Of the 46 dogs tested, 24 were male and 22 were female. The average age of dogs tested was 3.8±1.69 years. The dogs originated from 14 different North Island kennels. Three dogs were excluded as they were outside the age range. One dog was excluded due to lenticular anomalies.

Pedigree records were obtained through the NZKC website, after gaining pedigree names from owners. DNA samples were collected for all dogs, buccal swabs were collected for 8, and Oragene kits (infant collection version) were collected for 38 dogs. All DNA samples

were collected at the venue, apart from six which were collected by breeders and returned by post.

The distribution of the dogs tested from outside kennels is shown in Figure 10. A summary of the refractive error findings is detailed in Table 4. The two populations proved to have significantly different refractive error distributions (p=0.05)

	Case Population (GDS)	Control Population
n	180	46
Average SER (± SD)	-0.47±1.34D	+0.15±0.66D
Average anisometropia (% cases, range)	0.01D (5%, 1 to 1.49D)	0.04D (0%)
Astigmatism % (range)	4% (-0.50 to -2.60DC)	4% (-0.50 to -1.00DC)
Emmetropia (-0.49 to +0.99D)	55%	76%
Myopia (≤ -0.50D)	37%	15%
Hyperopia (≥+1.00)	8%	9%

Table 4 Refractive error findings in the two populations of Labrador Retrievers tested.

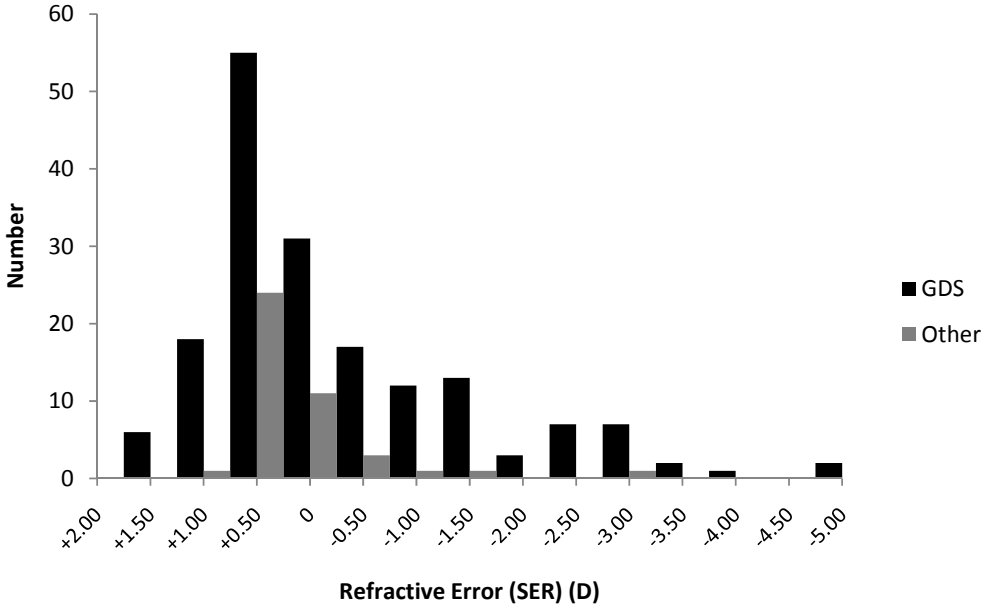


Figure 10 Distribution of refractive error in Labrador Retrievers from Guide Dog Services (GDS) south Auckland colony (n=180), and other breeders from throughout the North Island, New Zealand recruited for genetic association study (n=46)

3.4.2. Total Labrador Population

Overall the population (including all Labradors tested from GDS and other NZ breeders, n=226) was slightly myopic with an average SER of -0.41D. The average RE SER was -0.41±1.27D, and the average LE SER was -0.41±1.24D. Hyperopia was present in 6 % of the sample, Emmetropia in 60% of the sample and myopia in 34 % of the sample. The range of refractive errors was +2.82D to –5.37D (see Figure 11).

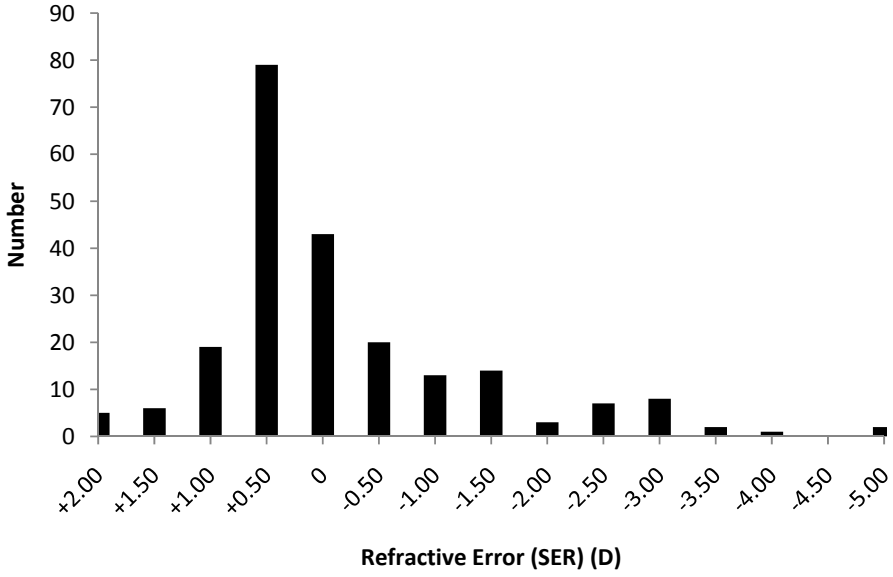


Figure 11 Distribution of refractive error in Labrador Retrievers in New Zealand, including all dog's tested (n=226).

3.4.3. DNA Sample Type

Following the small pilot study described in chapter 3.3.2, it was found that blood and buccal sample types amplified successfully, while toenail samples did not amplify.

Collection of toenail samples was also found to be problematic. In some dogs, who primarily lived indoors and were familiar with the procedure the nails were easily procured. However, in dogs that spent more time outdoors it was often difficult to collect a sample as the nail tissue was reduced, and collection could also induce an aggressive response. On the contrary multiple buccal swabs were easily collected. It was decided to continue collecting multiple buccal swabs and/or saliva samples.

3.4.4. DNA extraction and Preparation

A total of 165 DNA samples were collected and extracted. 120 samples were collected with buccal swabs (see Table 5) and 45 samples were collected using infant Oragene kits (see Table 6). The average yield with buccal swabs was 48.21±58.64 ng/µl (range 1.2-289.5ng/µl), and the average yield with Oragene kits was 81.72±76.73 ng/µl (range 8.9-257.8ng/µl). The difference in DNA yield between the two extraction techniques was statistically significant (p=0.01), with Oragene kits giving higher yields. Of the 90 samples identified for SNP array processing, 78 samples needed the concentration modified to 50ng/µl as specified in the Affymetrix manual (500K assay). 43 samples were concentrated using the Speedivac system, and 35 were diluted. 5 samples had to be excluded due to insufficient DNA yield, and were replaced with other cases/controls.

DNA purity as assessed by the A260/A280 ratio was significantly different (<0.01) on average between Oragene (saliva) (1.44) and buccal samples (2.12) (A260/A280 ratio of 1.5 or above indicates minimal protein contamination).

BUCCAL									
Sample #	Yield (ng/µl)	Total Yield(µg)	260/280	260/230	Sample #	Yield (ng/µl)	Total Yield(µg)	260/280	260/230
1	16.8	1.68	2.03	0.31	61	16.5	1.65	1.95	1.72
2	39.5	3.95	1.36	0.36	62	63.1	6.31	1.54	0.64
3	15.1	1.51	1.16	0.21	63	136.7	13.67	2.2	0.98
4	7.6	0.76	1.26	0.25	64	32	3.20	2	1.9
5	13.1	1.31	1.48	0.28	65	67.9	6.79	1.88	1.34
6	26.3	2.63	1.16	0.21	66	44.2	4.42	1.57	0.62
7	16.5	1.65	2.35	0.19	67	14.1	1.41	1.55	1.35
8	27.9	2.79	3.08	0.02	68	43.6	4.36	1.55	0.63
9	22.7	2.27	2.01	0.02	69	33.5	3.35	1.45	0.79
10	18.8	1.88	2.75	0.01	70	57.9	5.79	2.1	0.9
11	28.7	2.87	2.56	0.02	71	14.7	1.47	1.16	0.79
12	26.8	2.68	1.94	0.02	72	10.2	1.02	0.98	0.66
13	37.9	3.79	1.96	0.05	73	33.6	3.36	1.8	2.05
14	36.3	3.63	1.03	0.03	74	18.5	1.85	1.3	1.98
15	37.8	3.78	1.04	0.03	75	12.1	1.21	1.1	0.76
16	24.5	2.45	2.43	0.03	76	34.7	3.47	1.77	0.89
17	24.7	2.47	2.12	0.03	77	37.6	3.76	2.14	2.06
18	23	2.30	2.12	0.03	78	45.8	4.58	1.56	0.99
19	26.6	2.66	2.06	0.03	79	83	8.30	1.59	0.63
20	1.2	0.12	0.51	0.12	80	143.4	14.34	1.59	0.74
21	24.8	2.48	1.98	1.84	81	15.4	1.54	2.37	1.74
22	8.7	0.87	1.66	1.07	82	92.4	9.24	1.56	0.85
23	17.7	1.77	2.02	1.93	83	37	3.70	2.04	2.33
24	7	0.70	1.91	1.12	84	94.5	9.45	2.1	0.8
25	3.9	0.39	2.51	1.29	85	29.9	2.99	1.97	1.78
26	4.5	0.45	2.77	1.48	86	26.1	2.61	1.91	0.95
27	1.8	0.18	2.34	2.06	87	40.1	4.01	2.09	1.86
28	2.1	0.21	1.93	0.88	88	31.7	3.17	1.57	0.68
29	3.8	0.38	1.95	0.88	89	26.1	2.61	1.8	1.22
30	9.1	0.91	1.75	1.02	90	26.5	2.65	1.58	0.78
31	11.2	1.12	1.58	1.87	91	67.6	6.76	1.74	1.19
32	4.5	0.45	1.59	2.54	92	50.6	5.06	1.85	1.73
33	21.2	2.12	1.94	1.26	93	13.8	1.38	1.67	2.34
34	9.2	0.92	1.54	0.57	94	289.5	28.95	1.6	1.5
35	15.2	1.52	1.63	0.85	95	10.8	1.08	1.14	0.73
36	11.4	1.14	1.72	0.83	96	8	0.80	1.04	0.63
37	17.6	1.76	1.51	0.58	97	15.3	1.53	1.23	0.79
38	16.4	1.64	2.1	0.85	98	23.9	2.39	1.31	0.64
39	26.6	2.66	1.7	0.69	99	71.4	7.14	1.45	0.62
40	24.3	2.43	1.56	0.57	100	57.8	5.78	1.43	0.64
41	20.7	2.07	1.68	0.94	101	9.5	0.95	1.08	0.15
42	18.2	1.82	1.75	0.5	102	38.7	3.87	1.77	1.75
43	12.9	1.29	1.58	0.65	103	72.1	7.21	1.55	0.8
44	12.1	1.21	1.55	0.44	104	145.1	14.51	1.6	0.78
45	6.5	0.65	2.72	1.02	105	27.3	2.73	1.68	0.87
46	10	1.00	2.07	1.12	106	100.7	10.07	1.8	2.06
47	13.2	1.32	2.23	1.25	107	29.4	2.94	1.56	0.64
48	9.6	0.96	2.67	0.84	108	101.76	10.18	4.93	1.59
49	26.3	2.63	2.2	1.58	109	133.41	13.34	3.64	1.58
50	26.6	2.66	1.93	1.15	110	293.9	29.39	1.8	1.92
51	11.5	1.15	1.85	0.96	111	148.52	14.85	5.55	1.88
52	33.6	3.36	1.87	1.27	112	156.82	15.68	5.52	1.96
53	6.5	0.65	3.75	0.8	113	121.98	12.20	5.95	1.81
54	23.7	2.37	2.15	1.35	114	147.7	14.77	5.57	1.91
55	12.3	1.23	2.34	1.25	115	141.88	14.19	5.77	1.84
56	4.4	0.44	3.05	0.84	116	165.78	16.58	4	1.93
57	46.7	4.67	1.25	0.98	117	151.59	15.16	5.7	1.98
58	235.9	23.59	1.75	0.57	118	158.58	15.86	5.59	1.96
59	24.5	2.45	1.69	1.77	119	150.15	15.02	5.49	1.9
60	16.5	1.65	1.58	0.87	120	234.79	23.48	1.85	0.43

Table 5 Table summarising DNA extraction results for buccal swabs including concentration (ng/µl), total yield (in 100µl) and 260/280 and 260/230 ratios which are measures of DNA quality

ORAGENE									
Sample #	Yield (ng/µl)	Total Yield(µg)	260/280	260/230	Sample #	Yield (ng/µl)	Total Yield(µg)	260/280	260/230
2	11.4	1.14	1.26	0.45	**24**	37.4	3.74	1.27	1.47
3	56.6	5.66	1.33	0.25	**25**	54.1	5.41	1.51	0.55
4	10.4	1.04	1.7	0.57	**26**	38.8	3.88	1.18	0.53
5	21.6	2.16	1.57	0.68	**27**	94.1	9.41	1.67	0.39
6	26	2.6	0.95	0.15	**28**	47.4	4.74	1.57	1.61
7	8.3	0.83	1.09	0.17	**29**	47.2	4.72	1.4	0.31
8	38.3	3.83	1.85	1.55	**30**	44.5	4.45	1.44	0.72
9	55.9	5.59	1.55	0.83	**31**	74.2	7.42	1.3	0.65
10	8.9	0.89	1.44	0.48	**32**	34.9	3.49	1.37	0.37
11	45.3	4.53	0.95	0.18	**33**	15.2	1.52	1.29	0.36
12	37	3.7	0.82	0.83	**34**	126.4	12.64	1.49	0.34
13	158.6	15.86	1.59	0.48	**35**	57.3	5.73	1.48	0.46
14	160.7	16.07	1.75	0.18	**36**	28.1	2.81	1.41	0.46
15	132.4	13.24	1.59	0.13	**37**	223.5	22.35	1.78	0.77
16	14.4	1.44	0.88	0.59	**38**	257.8	25.78	1.9	1.18
17	67.9	6.79	1.48	0.74	**39**	208.4	20.84	1.6	0.53
18	121.6	12.16	1.65	0.68	**40**	107.9	10.79	1.53	0.43
19	63.7	6.37	1.55	0.25	**41**	270	27	1.74	0.72
20	38	3.8	1.24	1.3	**42**	298	29.8	1.55	0.5
21	31.2	3.12	1.73	0.55	**43**	195.4	19.54	1.41	0.35
22	117.3	11.73	1.56	0.64	**44**	29.5	2.95	1.32	2.25
23	24.8	2.48	1.3	0.36	**45**	31.2	3.12	1.49	1.08

Table 6 Table summarising DNA extraction results for oragene saliva kits including concentration (ng/µl), total yield (in 100µl) and 260/280 and 260/230 ratios which are measures of DNA purity

3.4.5. Relationship parameters

Relationship coefficients were calculated for all possible pairs of individuals within the case and control groups selected for the association study using the KinIncoef program (see Table 7 and Table 8). Kinship coefficients (described in chapter two) describe the probability of having an allele that is shared by two individuals due to a shared ancestor. A kinship coefficient of 0 defines no relatedness between two individuals.

The average kinship coefficient in the case population was 0.067(range 0-0.330). Ten percent of the individuals in the case population had no relatedness. The maximum value of 0.330 is equivalent to a relationship which is closer than parent-child (or siblings, value of 0.25) indicating a significant level of inbreeding. However, values greater than 0.25 were only found in 2% of cases.

The average kinship coefficient in the control population was 0.014 (range 0-0.281). Four pairs of individuals had greater than 0.25. Within the control dogs, 80% of pairs had a kinship coefficient of 0.

Inbreeding coefficients were also calculated across the population. Inbreeding coefficients are similar to the coefficient of relationship, except that they calculate the percentage of alleles which are shared due to ancestors which are common to both parents. Hence, an inbreeding coefficient is calculated for each individual rather than between pairs of individuals and is equal to the coefficient of relationship between the sire and dam of the dog.

The average inbreeding coefficient within the case population was 0.02 (range 0-0.148), and 44% had a value greater than zero, indicating some degree of inbreeding. In comparison average inbreeding coefficient in the control population was also 0.02 (range 0-0.125) and 30% of dogs had an inbreeding coefficient greater than zero.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	0.000																							
2	0.000	0.000																						
3	0.125	0.016	0.000																					
4	0.000	0.125	0.016	0.000																				
5	0.004	0.016	0.019	0.016	0.000																			
6	0.000	0.023	0.094	0.023	0.106	0.063																		
7	0.000	0.063	0.031	0.063	0.034	0.047	0.000																	
8	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000																
9	0.070	0.020	0.014	0.020	0.096	0.021	0.044	0.000	0.067															
10	0.063	0.008	0.016	0.008	0.050	0.070	0.016	0.000	0.073	0.000														
11	0.063	0.008	0.016	0.008	0.058	0.086	0.016	0.000	0.058	0.078	0.000													
12	0.000	0.047	0.031	0.016	0.050	0.078	0.031	0.000	0.014	0.016	0.031	0.000												
13	0.000	0.047	0.031	0.016	0.050	0.078	0.031	0.000	0.014	0.016	0.031	0.250	0.000											
14	0.000	0.047	0.031	0.016	0.050	0.078	0.031	0.000	0.014	0.016	0.031	0.250	0.250	0.000										
15	0.039	0.043	0.018	0.043	0.089	0.050	0.060	0.000	0.167	0.110	0.056	0.018	0.018	0.018	0.028									
16	0.031	0.066	0.016	0.066	0.025	0.047	0.039	0.250	0.031	0.066	0.035	0.016	0.016	0.016	0.057	0.000								
17	0.039	0.043	0.018	0.043	0.089	0.050	0.060	0.000	0.167	0.110	0.056	0.018	0.018	0.018	0.266	0.057	0.028							
18	0.000	0.031	0.063	0.031	0.100	0.156	0.063	0.000	0.027	0.031	0.063	0.250	0.250	0.250	0.035	0.031	0.035	0.000						
19	0.000	0.125	0.031	0.125	0.028	0.047	0.125	0.000	0.032	0.016	0.016	0.031	0.031	0.031	0.079	0.070	0.079	0.063	0.000					
20	0.000	0.094	0.016	0.094	0.015	0.023	0.063	0.000	0.018	0.039	0.008	0.016	0.016	0.016	0.081	0.051	0.081	0.031	0.188	0.000				
21	0.031	0.020	0.039	0.020	0.087	0.152	0.039	0.000	0.042	0.078	0.297	0.063	0.063	0.063	0.057	0.045	0.057	0.125	0.039	0.020	0.094			
22	0.031	0.020	0.039	0.020	0.087	0.152	0.039	0.000	0.042	0.078	0.297	0.063	0.063	0.063	0.057	0.045	0.057	0.125	0.039	0.020	0.313	0.094		
23	0.031	0.020	0.039	0.020	0.087	0.152	0.039	0.000	0.042	0.078	0.297	0.063	0.063	0.063	0.057	0.045	0.057	0.125	0.039	0.020	0.313	0.313	0.094	
24	0.000	0.000	0.000	0.000	0.125	0.125	0.000	0.000	0.000	0.000	0.063	0.125	0.125	0.125	0.000	0.000	0.000	0.250	0.000	0.000	0.094	0.094	0.094	0.000
25	0.031	0.047	0.008	0.047	0.077	0.031	0.031	0.000	0.040	0.039	0.027	0.027	0.027	0.027	0.086	0.033	0.086	0.055	0.094	0.125	0.031	0.031	0.031	0.078
26	0.000	0.016	0.031	0.016	0.047	0.063	0.031	0.000	0.058	0.031	0.141	0.047	0.047	0.047	0.036	0.016	0.036	0.094	0.031	0.016	0.109	0.109	0.109	0.063
27	0.063	0.133	0.031	0.133	0.051	0.094	0.078	0.000	0.062	0.133	0.070	0.031	0.031	0.031	0.115	0.133	0.115	0.063	0.141	0.102	0.090	0.090	0.090	0.000
28	0.125	0.016	0.031	0.016	0.069	0.141	0.031	0.000	0.084	0.125	0.125	0.031	0.031	0.031	0.080	0.070	0.080	0.063	0.031	0.016	0.141	0.141	0.141	0.000
29	0.063	0.020	0.039	0.020	0.079	0.090	0.039	0.000	0.156	0.051	0.066	0.133	0.133	0.133	0.053	0.035	0.053	0.266	0.039	0.020	0.088	0.088	0.088	0.125
30	0.063	0.020	0.039	0.020	0.079	0.090	0.039	0.000	0.156	0.051	0.066	0.133	0.133	0.133	0.053	0.035	0.053	0.266	0.039	0.020	0.088	0.088	0.088	0.125
31	0.008	0.079	0.006	0.016	0.099	0.040	0.036	0.000	0.142	0.038	0.038	0.053	0.053	0.053	0.132	0.011	0.132	0.074	0.024	0.014	0.041	0.041	0.041	0.125
32	0.008	0.079	0.006	0.016	0.099	0.040	0.036	0.000	0.142	0.038	0.038	0.053	0.053	0.053	0.132	0.011	0.132	0.074	0.024	0.014	0.041	0.041	0.041	0.125
33	0.004	0.047	0.081	0.047	0.100	0.153	0.096	0.000	0.105	0.070	0.066	0.089	0.089	0.089	0.116	0.051	0.116	0.178	0.090	0.046	0.137	0.137	0.137	0.031
34	0.063	0.066	0.016	0.066	0.043	0.023	0.039	0.000	0.164	0.039	0.039	0.016	0.016	0.016	0.069	0.053	0.069	0.031	0.070	0.051	0.035	0.035	0.035	0.000
35	0.031	0.066	0.016	0.066	0.041	0.047	0.039	0.000	0.062	0.191	0.051	0.016	0.016	0.016	0.096	0.129	0.096	0.031	0.070	0.082	0.053	0.053	0.053	0.000
36	0.031	0.066	0.016	0.066	0.041	0.047	0.039	0.000	0.062	0.191	0.051	0.016	0.016	0.016	0.096	0.129	0.096	0.031	0.070	0.082	0.053	0.053	0.053	0.000
37	0.063	0.055	0.023	0.055	0.042	0.082	0.047	0.000	0.051	0.082	0.066	0.023	0.023	0.023	0.112	0.061	0.112	0.047	0.109	0.133	0.080	0.080	0.080	0.000
38	0.063	0.055	0.023	0.055	0.042	0.082	0.047	0.000	0.051	0.082	0.066	0.023	0.023	0.023	0.112	0.061	0.112	0.047	0.109	0.133	0.080	0.080	0.080	0.000
39	0.000	0.063	0.078	0.063	0.050	0.117	0.125	0.000	0.042	0.070	0.039	0.078	0.078	0.078	0.081	0.051	0.081	0.156	0.125	0.156	0.098	0.098	0.098	0.000
40	0.000	0.063	0.078	0.063	0.050	0.117	0.125	0.000	0.042	0.070	0.039	0.078	0.078	0.078	0.081	0.051	0.081	0.156	0.125	0.156	0.098	0.098	0.098	0.000
41	0.063	0.258	0.023	0.070	0.043	0.082	0.047	0.000	0.052	0.129	0.066	0.039	0.039	0.039	0.093	0.100	0.093	0.047	0.078	0.055	0.080	0.080	0.080	0.000
42	0.063	0.258	0.023	0.070	0.043	0.082	0.047	0.000	0.052	0.129	0.066	0.039	0.039	0.039	0.093	0.100	0.093	0.047	0.078	0.055	0.080	0.080	0.080	0.000
43	0.000	0.031	0.063	0.031	0.068	0.156	0.063	0.000	0.027	0.063	0.063	0.063	0.063	0.063	0.051	0.047	0.051	0.125	0.063	0.031	0.125	0.125	0.125	0.000
44	0.000	0.031	0.063	0.031	0.131	0.281	0.063	0.000	0.027	0.125	0.125	0.063	0.063	0.063	0.082	0.078	0.082	0.125	0.063	0.031	0.219	0.219	0.219	0.000
45	0.008	0.032	0.037	0.032	0.125	0.118	0.067	0.000	0.156	0.077	0.069	0.053	0.053	0.053	0.161	0.039	0.161	0.105	0.056	0.030	0.119	0.119	0.119	0.063
46	0.008	0.032	0.037	0.032	0.125	0.118	0.067	0.000	0.156	0.077	0.069	0.053	0.053	0.053	0.161	0.039	0.161	0.105	0.056	0.030	0.119	0.119	0.119	0.063
47	0.008	0.032	0.037	0.032	0.125	0.118	0.067	0.000	0.156	0.077	0.069	0.053	0.053	0.053	0.161	0.039	0.161	0.105	0.056	0.030	0.119	0.119	0.119	0.063
48	0.008	0.024	0.068	0.024	0.108	0.087	0.052	0.000	0.149	0.046	0.046	0.053	0.053	0.053	0.141	0.019	0.141	0.105	0.040	0.022	0.060	0.060	0.060	0.125

Table 7 Table demonstrating kinship coefficients between the 50 dogs in the case group planned for association analysis. Inbreeding coefficients are included in columns which are self-referenced.

25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
0.000																							
0.018	0.000																						
0.066	0.031	0.031																					
0.039	0.031	0.141	0.000																				
0.053	0.080	0.070	0.102	0.031																			
0.053	0.080	0.070	0.102	0.266	0.031																		
0.034	0.040	0.023	0.014	0.054	0.054	0.000																	
0.034	0.040	0.023	0.014	0.054	0.054	0.250	0.000																
0.031	0.094	0.101	0.108	0.117	0.117	0.085	0.085	0.148															
0.049	0.041	0.105	0.078	0.143	0.143	0.037	0.037	0.057	0.008														
0.053	0.031	0.133	0.070	0.035	0.035	0.043	0.043	0.066	0.053	0.000													
0.053	0.031	0.133	0.070	0.035	0.035	0.043	0.043	0.066	0.053	0.254	0.000												
0.145	0.023	0.121	0.258	0.061	0.061	0.014	0.014	0.077	0.064	0.076	0.076	0.016											
0.145	0.023	0.121	0.258	0.061	0.061	0.014	0.014	0.077	0.064	0.076	0.076	0.258	0.016										
0.078	0.078	0.102	0.078	0.098	0.098	0.022	0.022	0.206	0.051	0.082	0.082	0.117	0.117	0.125									
0.078	0.078	0.102	0.078	0.098	0.098	0.022	0.022	0.206	0.051	0.082	0.082	0.117	0.117	0.313	0.125								
0.043	0.023	0.199	0.133	0.061	0.061	0.046	0.046	0.078	0.072	0.100	0.100	0.094	0.094	0.070	0.070	0.016							
0.043	0.023	0.199	0.133	0.061	0.061	0.046	0.046	0.078	0.072	0.100	0.100	0.094	0.094	0.070	0.070	0.258	0.016						
0.016	0.063	0.094	0.125	0.078	0.078	0.012	0.012	0.178	0.031	0.047	0.047	0.078	0.078	0.156	0.156	0.078	0.078	0.000					
0.016	0.063	0.156	0.250	0.078	0.078	0.012	0.012	0.209	0.031	0.078	0.078	0.141	0.141	0.156	0.156	0.141	0.141	0.250	0.000				
0.032	0.064	0.078	0.092	0.077	0.077	0.146	0.146	0.213	0.052	0.070	0.070	0.061	0.061	0.100	0.100	0.062	0.062	0.105	0.168	0.023			
0.032	0.064	0.078	0.092	0.077	0.077	0.146	0.146	0.330	0.052	0.070	0.070	0.061	0.061	0.100	0.100	0.062	0.062	0.105	0.168	0.277	0.023		
0.032	0.064	0.078	0.092	0.077	0.077	0.146	0.146	0.213	0.052	0.070	0.070	0.061	0.061	0.100	0.100	0.062	0.062	0.105	0.168	0.277	0.277	0.023	
0.037	0.056	0.039	0.029	0.073	0.073	0.159	0.159	0.125	0.044	0.051	0.051	0.026	0.026	0.061	0.061	0.027	0.027	0.043	0.043	0.165	0.165	0.165	0.012

Table 7 continued. Table demonstrating kinship coefficients between the 50 dogs in the case group planned for association analysis. Inbreeding coefficients are included in columns which are self-referenced.

	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
51	0.031																								
52	0.000	0.000																							
53	0.125	0.000	0.125																						
54	0.000	0.000	0.000	0.000																					
55	0.000	0.000	0.000	0.281	0.063																				
56	0.000	0.000	0.000	0.281	0.281	0.063																			
57	0.000	0.000	0.000	0.063	0.047	0.047	0.000																		
58	0.000	0.000	0.000	0.250	0.141	0.141	0.047	0.000																	
59	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000																
60	0.000	0.000	0.000	0.031	0.078	0.078	0.016	0.016	0.000	0.000															
61	0.000	0.000	0.000	0.031	0.141	0.141	0.016	0.016	0.000	0.188	0.000														
62	0.008	0.031	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000													
63	0.164	0.000	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.063												
64	0.004	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.039	0.006	0.000											
65	0.000	0.000	0.000	0.008	0.004	0.004	0.016	0.043	0.000	0.000	0.000	0.000	0.000	0.000	0.000										
66	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.125									
67	0.000	0.000	0.000	0.129	0.064	0.064	0.008	0.084	0.000	0.000	0.000	0.000	0.000	0.000	0.258	0.002	0.016								
68	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.125	0.000	0.063							
69	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.031	0.000	0.031	0.063						
70	0.000	0.000	0.000	0.000	0.000	0.000	0.047	0.023	0.000	0.000	0.000	0.000	0.000	0.000	0.078	0.000	0.039	0.000	0.000	0.063					
71	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.000	0.000	0.000	0.000	0.000	0.016	0.000	0.000	0.000				
72	0.008	0.031	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.250	0.012	0.039	0.000	0.000	0.000	0.016	0.000	0.000	0.125	0.000			
73	0.000	0.250	0.000	0.063	0.031	0.031	0.000	0.031	0.000	0.000	0.000	0.016	0.000	0.008	0.000	0.000	0.063	0.000	0.000	0.000	0.000	0.016	0.000		
74	0.000	0.094	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.070	0.000	0.051	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.070	0.047	0.000	
75	0.000	0.047	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.035	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.035	0.023	0.250	0.000
76	0.000	0.125	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.047	0.000	0.055	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.047	0.063	0.156	0.078
77	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
78	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.094
79	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.000	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.031	0.000	0.000	0.016	0.016	0.000	0.000	0.000
80	0.000	0.250	0.000	0.063	0.031	0.031	0.000	0.031	0.000	0.000	0.000	0.016	0.000	0.008	0.000	0.000	0.063	0.000	0.000	0.000	0.000	0.016	0.250	0.047	0.023
81	0.047	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.117	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000
82	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
83	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.000	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000
84	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000	0.016	0.000	0.000	0.004	0.133	0.002	0.188	0.031	0.000	0.016	0.016	0.000	0.000	0.000
85	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
86	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
87	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.031	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.031	0.031	0.000	0.000	0.000
88	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
89	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.001	0.008	0.203	0.004	0.094	0.031	0.000	0.000	0.000	0.000	0.001	0.000
90	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 8 Table demonstrating kinship coefficients between the 40 dogs in the control group planned for association analysis. Inbreeding coefficients are included in columns which are self-referenced.

76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
0.000														
0.000	0.000													
0.000	0.000	0.000												
0.000	0.016	0.000	0.000											
0.063	0.000	0.000	0.000	0.000										
0.000	0.000	0.000	0.000	0.000	0.063									
0.000	0.000	0.031	0.000	0.000	0.000	0.000								
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000							
0.001	0.000	0.000	0.031	0.000	0.000	0.000	0.063	0.063						
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000					
0.000	0.000	0.016	0.000	0.000	0.000	0.250	0.000	0.000	0.000	0.000				
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000			
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.250	0.000	0.000		
0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.281	0.000	0.000	0.000	0.000	0.000	
0.000	0.000	0.000	0.000	0.000	0.000	0.250	0.000	0.000	0.000	0.125	0.000	0.000	0.000	0.000

Table 8 continued. Table demonstrating kinship coefficients between the 40 dogs in the control group planned for association analysis. Inbreeding coefficients are included in columns which are self-referenced.

3.4.6. Sample Selection

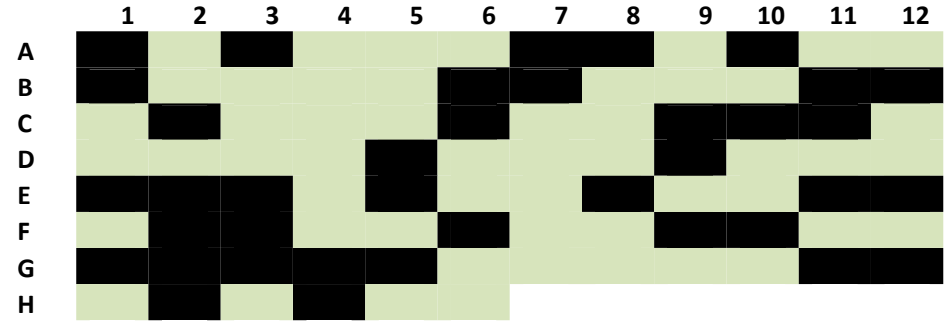
Following refractive error measurements and DNA extraction a small sample of dogs was selected for a pilot association analysis. The total sample consisted of 90 dogs, 50 myopic (case, ≤ -0.50D) and 40 non-myopic (emmetropic or hyperopic ≥-0.49D). Six dogs included in the initial case group were excluded due to insufficient DNA yield (as further samples could not be collected).

The case group had an average refractive error of -1.84± 0.49D with a range of -0.50 to -5.38D. The control group had an average refractive error of +0.41± 0.57D with a range of -0.44 to +2.00D.

Cases and controls were randomised prior to DNA processing. DNA was put into a 96 well PCR plate and sent to a commercial facility (Affymetrix) for processing prior to DNA hybridisation on the canine SNP arrays.

Figure 12 (a-c) below demonstrates the layout of the samples following randomisation.

Pilot Sample



a

	1	2	3	4	5	6	7	8	9	10	11	12
A	55	49.9	54.5	54.5	51.4	45.6	55.8	50	53.6	54.5	50.6	45.7
B	55.3	45.8	55.9	46.5	45.2	55.4	55.9	52.3	48.8	45.2	50.3	45.6
C	42.4	55.1	55.5	51.1	50.5	51	54.5	48.1	45.4	55.4	53	55.2
D	52.4	45.2	45.5	54.7	53.7	45	51.3	45.8	51.5	55.5	58.2	45.4
E	51.6	51.5	52.4	57.2	50.1	55	45.4	55.5	52.8	54.2	54.6	54.9
F	55	52	55.4	51.7	47.8	50.1	51.8	53.3	45.8	50.5	49	51.2
G	53.5	51.6	47.7	53.7	55.3	53.6	53.7	49.4	45.3	45	52.3	54.6
H	47	46.2	45.8	47.6	54.4	55.3						

b

	1	2	3	4	5	6	7	8	9	10	11	12
A	-2	-1.69	-1.69	+2	-1.25	-3.4	-1.62	+0.37	-3.25	-0.65	-0.59	-2
B	+1	-0.5	+0.37	+0.5	-1.25	-0.5	+1.68	-1	+1.5	+0.37	+1.88	+0.75
C	-1.88	+0.75	+0.25	0	-2.13	-1.13	-2.81	-1.64	0	0	+0.5	0
D	-3.75	-1	-0.63	+0.5	-1	-0.56	-2.66	-0.94	+0.12	-2	-0.88	-0.94
E	-0.25	0	-2.87	0	-0.75	-5.38	-0.25	+0.37	0	-1.88	-3.63	0
F	0	-1	-2.66	-2.5	-2.66	+0.82	+0.5	+0.12	+0.37	-1.12	-0.94	-2
G	-5.25	+0.37	-0.88	+1.12	-0.44	-3.31	-2.81	-3.25	-1	+0.25	+0.12	+0.06
H	-0.75	-1.13	0	+0.5	-1.94	0						

c

Figure 12 The layout of the final sample to be included in the whole genome association analysis as processed in a 96 well plate. The order of samples was randomised prior to plating of DNA. (a) demonstrates DNA types across the sample O-Oragene B-Buccal (b) demonstrates DNA concentration across the sample and (c) demonstrates case and control distribution across the sample. Cases are represented in dark squares, and controls are represented by light squares, the refractive error for each dog is shown.

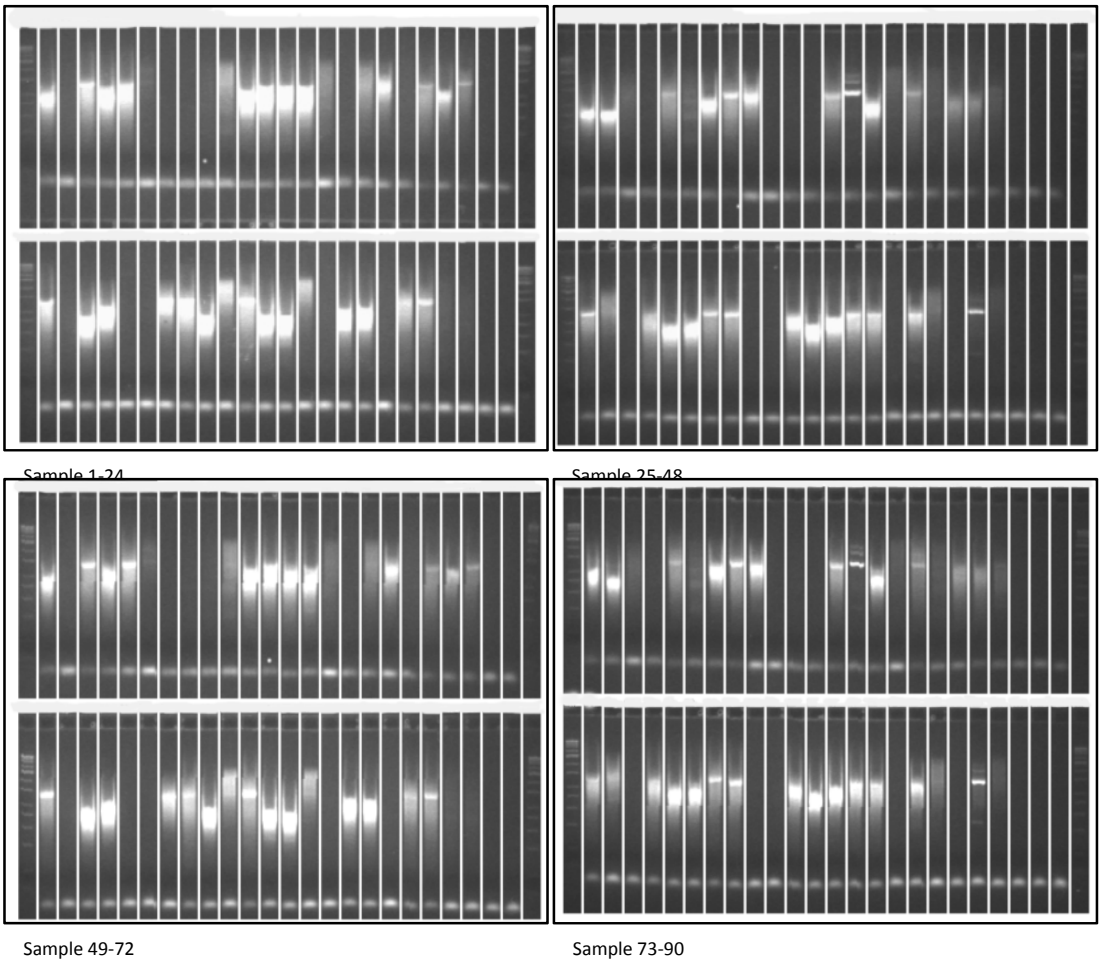


Figure 13 Gel images of DNA product following PCR amplification, dark bands represent amplification failure.

3.4.7. Sample Processing

Following the establishment of a myopic case and a non-myopic control population, extraction of DNA and calculation of necessary relationship parameters; sample processing for association analysis was performed by the University of Auckland Affymetrix facility. As previously described (chapter 3.3.7), this was by the protocol for the v2 canine SNP array. Samples were streamed through the first three steps of the process. However, during the amplification stage (step 3) using the STY marker, there was a high rate of amplification failure. Only 46 of the 90 samples were amplified successfully (Figure 13). There was no significant difference in the rate of successful amplification between samples extracted from buccal or Oragene samples. There was a slightly higher rate of amplification failure in the case vs. the control populations.

3.4.8. Future Association Analysis

As there was such a significant level of amplification failure, the DNA was not processed to the point of hybridisation on the chips. The SNP arrays involved in this study are able to kept refrigerated for several years, so it was decided to optimise the sample before proceeding. The future plan for the study is to continue to collect DNA samples and refractive errors in Labrador Retrievers to improve the relatedness within the case and control populations, and to increase the number of moderately myopic dogs (≤-2.00D).

Chips will be analysed using the Affymetrix Genotyping Console software to make allele calls (AA, AB or BB) at each SNP. The data will then be put into standard linkage format and analysed using PLINK software to analyse standard summary statistics (for quality control) such as missing genotype rates, minor allele frequencies and Hardy-Weinberg equilibrium failure rates (Purcell, Neale et al. 2007). Association analysis will then be completed using MQLS software, which has been specifically designed for association testing with case-control groups involving a level of relatedness (Thornton and McPeek 2007).

3.5. Discussion

3.5.1. Refractive Error Distributions between case and control populations

As can be seen in Figure 10 there are different refractive error distributions between the case and control populations. The case group had a much higher prevalence of myopia than previously reported in groups of Labrador Retrievers (Murphy, Zadnik et al. 1992; Kubai, Bentley et al. 2008). In comparison, the control population sourced from other New Zealand breeders had a range of refractive errors very similar to those previously reported (Kubai, Bentley et al. 2008).

It should be noted that although the case group of dogs tested in the present study were part of a service dog breeding colony, the majority of dogs that were tested were not working guide dogs. Most dogs tested were in the training stage, had been withdrawn (for health or temperament reasons) or were breeding stock. This may have biased the proportion of myopia found in the pedigree overall. However, dogs were tested from the majority of litters within the pedigree, as it was rare to find a litter where all puppies had proceeded to graduate from training. To get a more accurate estimation of the refractive error distribution in the pedigree it would be beneficial to test a cohort of working guide dogs, as the high level of myopia detected primarily in withdrawn dogs may be intrinsically linked to their removal from training due to behavioural factors such as anxiety and reluctance to walk.

3.5.2. DNA collection and extraction

One of the initial challenges with carrying out a genetic study in dogs was the best way of sourcing DNA. Most studies investigating canine disease use blood samples, however these are usually sourced through veterinarians who refer dogs to study centres and provide blood samples. Because in the current study the investigators were phenotyping dogs and collecting DNA samples, the mode of collection needed to be rapid, portable and as non-invasive as possible. When the study began there were mixed reports on the use of buccal swabs in canine studies with some studies reporting successful extraction and acceptable yields (Oberbauer, Grossman et al. 2003; Chang, Terrill et al. 2007) and others reporting that this sample type gave inadequate yield for successful amplification (Short, Kennedy et al. 2005).

It has also been reported that buccal samples tend to have a high bacterial component in human studies (Herraez and Stoneking 2008).

In comparison buccal and saliva samples have been used widely in human studies over the last several years (Borthakur, Butryee et al. 2008). In conjunction with this increase in popularity of buccal and saliva sample collection, there has been a rapid increase in the number of commercial kits available for sample collection and extraction in human subjects. When we began the study there were fewer options available tailored for use with animals. Several DNA sample types were trialled initially to find the best solution in terms of ease of collection, time taken, invasiveness and portability. Buccal and saliva samples ended up providing the best solutions in terms of the above points. Having now collected samples in a large number of dogs, there seem to be several factors which influence yield and quality which differ from the manufacturer's recommendations. There was also large variation in the amount of saliva that each dog secreted, and the amount was particularly low following refractive error testing as the dogs tended to be anxious.

The main factor which influences DNA yield is the amount of high quality sample collected. However it is difficult to get a sufficient amount of a sample in a short period of time. One way to increase the yield is to sample numerous times over longer time periods. Ideally, the dog owner would be given several kits or swabs that they could collect over several hours/days. The recently released animal Oragene kits have recommended that sponges remain in contact with the dogs' mouth for at least 30 seconds. This length of time is very difficult to achieve in a single sampling period. As with human kits, free saliva collected directly into the vial would give the largest yield of cells and be the least contaminated. As dogs, and particularly Labradors, are so receptive to the visual and olfactory signals associated with food, one way of increasing the amount of sample would be to collect saliva prior to meal times or show the dog food to elicit salivation during sample collection.

In summary, we have demonstrated that both saliva and buccal samples are easily obtained from dogs, are portable and non-invasive. Saliva samples using the Oragene collection system seems to give greater yields than samples collected with buccal swabs. Although there was a relatively high amplification failure rate, this was on a single sample. If multiple samples were taken over an extended period these methods represent an ideal DNA sample type in dogs.

3.5.3. Relationship Parameters

Although some level of relatedness is beneficial in genetic studies, it is essential to have case and control populations that are as unrelated as possible. One difficulty which has arisen in the current project is the limited population of dogs available in New Zealand. Although Labrador Retrievers are the most popular breed of dog in New Zealand, most pedigrees going back 4-5 generations still originate from the same founding individuals. The Guide Dog Colony has had multiple dogs donated from the other major kennels around New Zealand and shares breeding stock with Australian Guide Dog Schools. This level of relatedness reduces the number of individual genomes within the samples, and also makes it more difficult to detect genetic differences between affected and unaffected groups. It is inevitable that within a pedigree population there will be a high level of shared genetic code, even across different countries. However, if dogs could be sourced from different founding individuals these commonalities could be minimised. Optimally the case population would be sourced from one population such as New Zealand GDS and the control population could be taken from another source with a larger number of breeders such as Australia. Having said that, there was no relatedness in only 10% of GDS with 2% of high relatedness and 44% of inbreeding, compared to 80% of the control population that were not related and only 30% of inbreeding.

Following the unexpectedly high rate of amplification failure prior to DNA hybridisation on the canine arrays, SNP analysis was unable to be undertaken as it would yield results that were not statistically sound. These low yield rates of DNA amplification indicate that future studies need to utilise a modified protocol. Possibly by collecting greater sample sizes (for example Oragene saliva samples (2mL) collected over 5 consecutive days and then pooled) to maximise DNA yield and purity. This was not possible in our sample due to low success rates in owners returning kits and older dogs dying etc. To further optimise the sample, a greater myopic population should be recruited. Ideally, the relatedness of the control population could also be decreased to improve statistical power, although an 80% non-relatedness quotient is low for pedigree dog populations. To improve the control population a separate group of dogs could be tested in Australia or the South Island of New Zealand to reduce the relationship coefficients.

3.5.4. Future Directions

Having established viable solutions for extracting DNA using non-blood sources, accurate ways of calculating relationships and having established that ametropia is prevalent in canine

populations, they prove to be a valid species for further studies at a genetic level. Although the association analysis was unable to be conducted with statistical soundness in the study population described in this chapter, all the necessary techniques and parameters have been determined to complete such a study.

Much like in human myopia genetics studies, it would be extremely important in future canine association analysis studies to rule out systemic disease genotypes which may predispose the dogs to myopia. As there is such a high prevalence of skeletal dysplasia in Labrador Retrievers, as detailed in chapter four, it would be important to rule out a sub-clinical case of this disorder. It may be that an allele predisposes these dogs to both conditions. To do this, other clinical measurements such as hip scores (a numerical value which is used to define the degree of hip dysplasia following hip x-rays) could be analysed during genotyping to determine if there was any association. It would also be of interest to analyse any association between hip scores, which show a tendency towards hip dysplasia, and myopia. Skeletal dysplasia is undoubtedly associated with high myopia in multiple human genetic syndromes.

One unexpected hurdle that was encountered when trying to establish a control population was the reluctance of NZ breeders to have their breeding populations phenotyped for refractive error. Although all breeders were contacted around the North Island the response rate was lower than 10% (3 out of 35 possible breeders). There seemed to be a common assumption among breeders that we were looking for ocular pathology as well as refractive error. Retinal dysplasia, a common condition in LR (described in chapter four) is still occurring in New Zealand LR populations due to the lack of a genetic registry, which has become common practice in other countries. There is now a genetic test which is available commercially which tests for the causative gene for this disease. Breeders that have had this test have 'certified' breeding dogs that are free of the gene. All breeders that had had their dogs certified participated in refractive error testing, and were interested in participating in the study. However, there was a common assumption that if we looked for the gene for refractive error, we may also look for the gene for retinal disease. Any discovery of this gene in their breeding animals, even if not at a clinical level would require them to declare that their animals were affected, which would in turn influence the future value of their puppies.

Chapter 4. Chapter Four – Environmental Factors

Summary of Chapter Four

In chapter two, the level of myopia was shown to be higher in dogs from smaller litters (1-8 puppies) when compared to dogs from larger litters (9-13 puppies). Following this result further investigation into early environmental influences that could be affected by litter size were indicated. There are several factors which could influence the rate and extent of development of myopia and therefore have an effect on adult refractive error. Pre-natal environments could differ depending on factors such as maternal heath, previous obstetric history, maternal age, size of litter and length of gestation. The post-natal environment could be influenced by factors such as competition for nutrition, number of littermates, outdoor activity, light levels, temperature, health during the first months of life, age of weaning and type of nutrition. One of the advantages of this study population was that many of these factors were kept reasonably constant between all litters. All dogs were provided with the same food and kept within the same weight range, and all breeding animals had all veterinary care provided. During pregnancy, brood bitches were supplied with the same nutrition and exercise regimes. All puppies were born in the same breeding facility in Auckland and kept there until 6 weeks of age. The main factors which differed between litters were sire and dam, season of birth, litter size and litter cohort. These factors were studied in terms of their influence on adult refractive error in the large pedigree of Labrador Retrievers studied in chapter two. Other factors such as the influence of season of birth on litter size and birth weight were studied on all available Labrador Retriever puppies within the GDS colony.

4.1. Introduction

4.1.1. Effects of Season and Photoperiod – Human and Animal Studies

4.1.1.1. Human Disease Prevalence and Seasonality

Season of birth influences the development of numerous human conditions including schizophrenia (Kirkpatrick, Messias et al. 2008), bipolar disorder (Dassa and Azorin 1993), adult obesity (Wattie, Ardern et al. 2008), as well as myopia (Mandel, Grotto et al. 2008). The current theories explaining seasonal differences in disease prevalence are that different exposures to environmental influences such as light and temperature at birth affects disease course, however the exact causative factors are still unknown. There is evidence that people born in winter are more likely to suffer from conditions caused by changes in tissue derived from embryonic ectoderm destined to become nervous tissue, whereas those born in summer are more likely to have conditions related to embryonic ectoderm that becomes gut, lung and glandular organs (Davis and Lowell 2006). Apart from the effect of season of birth there is a wide base of evidence to show that diseases, including autoimmune, infectious and cardiovascular conditions are more likely to present and recur in certain seasons (Chougnet, Deloron et al. 1990; Tang, Santella et al. 1995).

Birth season also effects birth weight; humans tend to have a higher birth weight in winter and spring compared to summer and autumn (Murray, O'Reilly et al. 2000; McGrath, Barnett et al. 2005). Pre-term births also show a seasonal effect, with more pre-term babies born in developed countries in summer (Keller and Nugent 1983; Cooperstock and Wolfe 1986) and winter relative to spring and autumn (Northern Hemisphere) (Matsuda and Kahyo 1992; Lee, Steer et al. 2006; Bodnar and Simhan 2008). A British study reported a 10% higher chance of a baby being born pre-term if born in winter compared to spring (Lee, Steer et al. 2006). The effect of season of estimated conception has also been investigated, with conceptions in late summer and early autumn resulting in the lowest number of preterm births while conceptions in early spring present with the highest number of preterm births (Bodnar and Simhan 2008).

Term of gestation and birth weight have been shown to affect refractive outcome in children, and these factors have been hypothesised to explain the seasonal variation seen in refractive error according to birth season (Varughese, Varghese et al. 2005; McMahon, Zayats et al. 2009). Babies that are born at full-gestational term (defined as 37 completed gestational weeks) are slightly hyperopic on average (Kuo, Sinatra et al. 2003; Varghese, Sreenivas et al.

2009). However, babies born prematurely (defined as less than 37 completed gestational weeks) have more myopic refractions at equivalent full-term, and the level of myopic shift increases in relation to the level of prematurity (Holmstrom, el Azazi et al. 1998; Quinn, Dobson et al. 1998; Choi, Park et al. 2000; Saunders, McCulloch et al. 2002). However, a recent study has shown that birth weight seems to be a more reliable indicator of refractive status than gestational age or other anthropomorphic indicators such as head circumference or body length (Varghese, Sreenivas et al. 2009). Dirani et al (Dirani, Islam et al. 2009) investigated the role of birth weight in refractive error development as part of the GEM (genes in myopia) study, investigating monozygotic and dizygotic twin pairs but they found no role for birth weight as a causative factor in myopia development in twins

4.1.1.2. Season of Birth and Adult Refractive Error

The link between birth season, other environmental factors and adult refraction has only recently been investigated. Mandel et al investigated season of birth, photoperiod and refractive error in a group of Israeli conscripts and found a correlation between photoperiod and myopia, with significantly higher levels of myopia in conscripts born in spring and summer (Mandel, Grotto et al. 2008). McMahon et al (McMahon, Zayats et al. 2009) conducted a similar study in a UK population, with data gathered from optometry practices, and also found significantly more high myopes being born in summer or autumn. However the study did not agree with the previous findings that photoperiod affected myopia level, instead suggesting that the higher level of myopia in summer and autumn may be due to other factors such as birth weight which also has seasonal fluctuations (McGrath, Barnett et al. 2005).

4.1.1.3. Effect of Night Lighting and Photoperiod – Human studies

There have been varying reports about the effect of using "night lights" on myopia development in children. Quinn et al first reported this effect in 1999, finding that children who slept with full room lighting were approximately five times more likely to be myopic compared to children who slept in darkness (Quinn, Shin et al. 1999). In response to this publication, later studies did not find any association between ambient night lighting and myopia (Gwiazda, Ong et al. 2000; Zadnik, Jones et al. 2000; Saw, Wu et al. 2001). However, these studies did find that myopic parents were more likely to use night lights with their children, perhaps enhancing the parents' vision at night. A study investigating a cohort

from the UK also found no relationship between night lighting and myopia, but did find that myopic parents were more likely to have myopic children, and patients of Asian descent were more likely to be myopic (Gwiazda, Ong et al. 2000; Guggenheim, Hill et al. 2003). Chapell et al also reported no increase in the prevalence of myopia in children who slept with lighting between 0-2 years of age, but did find that adults who were reported to have slept with night lighting during this period by their parents were significantly more likely to be myopic (Chapell, Sullivan et al. 2001).

It has also been found that Finnish conscripts living above the arctic circle, where photoperiods are at an extreme, display higher levels of myopia than their colleagues living further south, but did not find an association between season of birth and myopia (Vannas, Ying et al. 2003).

In summary, there is substantial evidence to support the idea that the development of many diseases is influenced by season of birth. The onset and recurrence of diseases also seems to follow a measurable seasonal fluctuation. Myopia seems to be a condition that is influenced by season of birth, with increased myopia risk, particularly for moderate and high myopia if born in summer or autumn (Mandel, Grotto et al. 2008). There has been a number of studies investigating the effect of photoperiod, in relation to night lighting with infants which have showed mixed results. Some studies found an increased risk of myopia development if children were exposed to night lighting during early childhood (Quinn, Shin et al. 1999; Chapell, Sullivan et al. 2001), while other studies found no association (Gwiazda, Ong et al. 2000; Zadnik, Jones et al. 2000; Saw, Wu et al. 2001). There is also evidence that people who live in naturally extreme lighting conditions exhibit higher myopia prevalence rates (Vannas, Ying et al. 2003).

4.1.1.4. Outdoor Activity and Light

Light exposure later in childhood also seems to have an affect on myopia development in association with outdoor exercise. This association was found (Rose, Morgan et al. 2008) in 6 and 12 year old Australian children who were questioned on their levels of indoor and outdoor exercise, reading and near work levels and family history of refraction. Children who spent more time outdoors demonstrated more hyperopic refractive errors, independent of the amount of near work they were involved in or their family history of myopia. This finding was supported in a parallel study of children from Singapore where levels of myopia were lowest in children who performed more outdoor activity (Dirani, Tong et al. 2009). Jones et

al also found that sport and outdoor activity was an important predictor for myopia onset, finding that outdoor exercise and sport decreased the likelihood of becoming myopic, particularly when the child had two myopic parents (Jones, Sinnott et al. 2007). These findings were not replicated in a study of Chinese children, which found no relationship between increased time outdoors and myopia. Interestingly they also found no relationship with near-work (Lu, Congdon et al. 2009). However, the children Lu et al questioned were older (average age of 14 years of age) and demonstrated a much higher prevalence of myopia (81%) than that observed in the Australian sample (Rose, Morgan et al. 2008; Lu, Congdon et al. 2009).

A similar investigation (Deere, Williams et al. 2009) went on to investigate activity levels in adolescents directly with the use of an accelerometer (UK cohort), measuring the number of minutes they engaged in moderate to vigorous activity both indoors and outside. It was found that myopic children engaged in lower levels of activity and were more sedentary compared to other refractive error groups. However, the location of the activity was not reported, so there was no report of indoor/outdoor activity.

4.1.1.5. Effect of Photoperiod – Animal studies

The effect of photoperiod on post-natal eye growth has previously been studied in a variety of animal species including chicks and primates (Stone, Lin et al. 1995). Constant light exposure seems to alter ocular growth signals in both lens induced and form deprivation myopia, leading to more hyperopic refractive errors due to corneal flattening (Guo, Sivak et al. 1996). Chicks raised in constant light exhibit inhibition of anterior segment growth, while also showing increased vitreous chamber elongation (Li, Troilo et al. 1995). These changes are not observed in light-reared infant primates, which exhibit the low hyperopic refractive error seen in control animals, however there is limited evidence that emmetropisation mechanisms may not work as efficiently as in those with a clear light-dark cycle (Smith, Bradley et al. 2001).

4.1.1.6. Effect of Dopamine on Myopia Development

Dopamine is a monoamine neurotransmitter which is found in amacrine or interplexiform retinal cells in all vertebrate species, and has a neuromodulator-activity in the retina (reviewed by (Brandies and Yehuda 2008). Although only a small proportion of amacrine cells are dopaminergic, the complex network of connections provides coverage across the

retina. Dopamine has been shown to have similar localisation, modes of actions and roles in visual processing across vertebrate species. Dopaminergic receptors are found on all morphological retinal neuron types (Brandies and Yehuda 2008). Dopamine has an important role in retinal light adaptation, emphasising the light driven cone input and attenuating the rod input (Witkovsky 2004).

Altering dopamine levels provides a possible explanation for the effect of photoperiod on myopia. Diurnal dopamine fluctuation influences retinal dark-light adaptation, as well as influencing eye growth through presynaptic D1 receptors (Witkovsky 2004) . There is a suggestion that dopaminergic amacrine cells may be involved with visually guided eye growth rather than just in response to myopia induction such as in FDM (Pendrak, Nguyen et al. 1997). Atropine has been shown to reduce the progression of myopia in children (Saw, Gazzard et al. 2002), which apart from blocking accommodation atropine also increases the release of dopamine; which may alter eye growth signals (Schwahn, Kaymak et al. 2000).

Animal studies have shown that the levels of retinal dopamine, tyrosine hydroxylase (an enzyme involved in the synthesis of dopamine) and DOPAC (3,4-dihydroxyphenylacetic acid, the principal metabolite of dopamine) are reduced during developing deprivation myopia (Stone, Lin et al. 1989; Iuvone, Tigges et al. 1991). The reduction of tyrosine-hydroxylase and DOPAC indicates that dopamine synthesis and release may be involved in the development of myopia. The activity of dopamine has been shown to be greatest during the day, with increasing light levels causing dopamine synthesis and release. Maximum DOPAC levels are observed within 3 hours of light exposure in chicks (Luft, Iuvone et al. 2004). However, these levels are reduced in the presence of form deprivation (Megaw, Morgan et al. 1997).

When FDM is induced in chicks, retinal dopamine and DOPAC levels are reduced in occluded eyes when compared with control and recovering eyes (Pendrak, Nguyen et al. 1997). When goggles were removed and the eyes were allowed to recover, a gradual increase in dopamine, DOPAC and the DOPAC/dopamine ratio (an index of dopamine metabolism) was seen until comparable with control eyes.

Applying a dopamine (or dopamine/serotonin) agonist (apomorphine, reserpine) reduces the level of FDM in chicks (Stone, Lin et al. 1989; Iuvone, Tigges et al. 1991; Schaeffel, Bartmann et al. 1995), and prevents FDM in rabbits (Gao, Liu et al. 2006).

The finding that atropine reduced myopia development in studies of FDM in animals and reduced the rate of myopia progression in clinical trials of children led to trials of other muscarinic antagonists. Clinical trials have (Siatkowski, Cotter et al. 2008) successfully reduced the progression of myopia by almost half through the application of pirenzepine (an M1 selective muscarinic antagonist) ointment over a 2 year period.

The turnover of monoamine neurotransmitters has been shown to be affected by season with dopamine turnover being highest in those born in November-December (NH) and lowest in those born in March (Chotai and Adolfsson 2002). This as previously mentioned supports season as being an important factor in the development of conditions such as myopia, and raises the possibility of variation in neurotransmitter turnover being a factor in refractive error development.

4.1.2. Canine Breeding Parameters

4.1.2.1. Duration of Gestation

Gestation length varies between different breeds of dogs, with shorter gestation durations in larger breeds (Eilts, Davidson et al. 2005). Other factors which influence gestation length are age of the bitch, litter size and parity (technical term that defines the number of times a female animal has given birth) (see glossary for expanded definition) of the litter. Various patterns for these parameters have been reported, but the largest study of Labrador Retrievers to date (Eilts, Davidson et al. 2005), which investigated 482 whelping events found that gestation was shorter in Labrador Retrievers compared to German Shepherds, Golden Retrievers and a group of hounds. Gestation was also found to be influenced by litter size, with litters of four or less having a significantly longer gestation period than litters of five or more. It was also found that age and parity had no effect on gestation duration.

4.1.2.2. Birth Weight

There is little published research on average birth weights for different dog breeds. One study gives the average birth weight of LR as 401g (SE=14.1) for males and 392g (SE=9.2) for females (Helmink, Shanks et al. 2000). Heritability of birth weight is reported to be 0.24 (Helmink, Rodriguez-Zas et al. 2001).

4.1.2.3. Growth Rate

Large breed dogs tend to reach their maximum adult weight at between 300 to 480 days (10-16 months) of age (Helmink, Shanks et al. 2000), and achieve 50% of this weight by around 4 months of age. As LR and GS tend to be the most commonly used guide dogs, most studies have looked at growth curves within these breeds. Males (LR & GS) tend to be 4.7 ±.2 kg heavier than females at full adult weight (Helmink, Shanks et al. 2000) with the average mature weight of LR being 25kg for females and 30kg for males (Helmink, Shanks et al. 2000). Weight gain seems to reach its peak at 89-106 days as measured by the Gompertz growth function (Trangerud, Grondalen et al. 2007), after which time it slowly plateaus until adult weight is achieved. There seems to be a significant association between weight at 42 days and adult mature weight, selecting heavier puppies at 42 days would result in an average heavier adult weight (Helmink, Rodriguez-Zas et al. 2001). Heritability estimates of adult mature weight were found to be 0.57 ±0.07 for GS and 0.44 ±0.07 for LR. Positive genetic correlations were also found for mature weight and mature height (Helmink, Rodriguez-Zas et al. 2001).

4.1.2.4. Seasonal effects on canine fertility

Since the dog was domesticated 15,000 years ago, the rate of fertility has increased. Compared to their ancestor, the wolf, which has one female oestrus cycle per year (January to March (northern hemisphere)), female dogs (bitches) have two to three oestrus cycles, meaning that domestic dogs are considered to be non-seasonal or mono-oestrus breeders.

Fertility in a range of species including pigs, cows and dogs, varies by season, possibly due to changes in temperature and light levels (Chatdarong, Tummaruk et al. 2007). The reproductive patterns of canids differs from other mammalian species in that each oestrous period is followed by a long interestrous interval (diestrus and anoestrus), producing a reproductive cycle described as monocyclic (Chatdarong, Tummaruk et al. 2007). Breeding patterns also differ between different dog breeds, with breeds such as the Spitz preferentially breeding in Autumn (Tedor and Reif 1978). Studies also suggest that overall more females come into estrous during the winter and spring compared to autumn and particularly summer (which correlates to the wolf), although these patterns differ according to geographic location. A study in Quebec, Canada showed that more oestrous periods were observed in domestic dogs during summer and winter (Bouchard, Youngquist et al. 1991), in comparison to Britain where more oestrous periods were observed during the spring (Christie and Bell

1971). In more tropical climates (India) a greater number of oestrous periods were observed in stray dogs in late autumn and winter (Chawla and Reece 2002). Feral dogs show clearer seasonal patterns in breeding when compared to domesticated dogs (Christie and Bell 1971), with wild dogs reverting to feral behaviours where only the alpha male and female breed, and other males assist in the rearing of pups (Ortega-Pacheco, Segura-Correa et al. 2006).

4.1.2.5. Seasonal effects on litter size

Season of birth also affects litter size, with reduced litter size in seasons with higher ambient temperatures. In a study looking at fertility in a colony of Labrador Retrievers and German Shepherds in Thailand (Chatdarong, Tummaruk et al. 2007), where the light levels remain fairly constant throughout the year, it was found that season affected whelping rates (refers to the number of matings which lead to successful whelping). Litter size was found to be higher in March (spring) than in September (autumn). There was also a greater level of neonatal puppy death in September. At the other end of the spectrum, a similar study investigated the effect of season on reproductive patterns in a breed of hunting dog in Sweden where day (photoperiod) ranges from 0-18h (Gavrilovic, Andersson et al. 2008) found that the fewest whelpings occurred in summer and the largest litters were born in spring. Litter size was also found to be affected by gestation. Parity and age of the bitch also showed effects on mating success and litter size (Chatdarong, Tummaruk et al. 2007).

4.1.3. Canine Ocular Pathology

4.1.3.1. Cataracts

Cataracts are a common condition in dogs, particularly in Labrador Retrievers. In the dog, the lens contributes approximately 40D to the power of the eye, which is 60D in total (Davidson, Murphy et al. 1993). After cataract removal, it has been predicted that vision in the aphakic dog drops to around 20/800 or worse (Murphy, Mutti et al. 1997). Cataracts display a heritable component but a mode of transmission or genetic locus has not yet been identified in this breed. Cataracts also seem to co-exist with other ocular pathology such as progressive retinal atrophy. The most common type of cataracts is posterior polar (triangular) cataracts (PPC), which is usually detectable by 2 years of age. Although this type of cataract is assumed to not cause visual impairment by many breeders, PPC has been shown to progress in 20% of Labrador and Golden Retrievers (Curtis 1989). The prevalence of

cataracts in Labradors has been reported as between 5.5-8% depending on the study population (Curtis 1989; Kraijer-Huver, Gubbels et al. 2008).

4.1.3.2. Retinal Dysplasia

Retinal dysplasia is due to a malformation of the retina during development caused by the two primitive layers of the retina not forming together correctly. Mild dysplasia manifests as folds in the inner retinal layer, clinically termed 'retinal folds'. In the more severe form of the disease 'geographic dysplasia', the two layers of the retina do not form together causing a detachment from the retinal pigment epithelium at birth or within the first six weeks of life, the time period when the canine retina goes through the majority of postnatal retinal maturation (Holle, Stankovics et al. 1999). Retinal dysplasia is not progressive, so that clinical signs seen at birth do not worsen. Examination can be conducted as early as six weeks to detect retinal dysplasia. The aetiology is most commonly genetic, but can also be due to pre-natal infections such as parvovirus or herpes. The mode of inheritance has not been identified across all breeds, but has been identified as autosomal recessive in a subset of breeds. 25 breeds of dog have been reported to suffer from retinal dysplasia. Some breeds, notably Labrador Retrievers and Samoyeds (Meyers, Jezyk et al. 1983; Holle, Stankovics et al. 1999) can have concurrent skeletal and retinal dysplasia, termed oculoskeletal dysplasia. This condition is thought to be autosomal dominant in causation, with homozygous dogs suffering from both skeletal and ocular changes, whilst heterozygous dogs show only retinal changes (Carrig, MacMillan et al. 1977; Acland and Aguirre 1995).

4.1.3.3. Systemic disorders – Skeletal Dysplasia

Skeletal dysplasia is very common in large dog breeds and can occur in isolation or with associated retinal changes. Hip dysplasia is the most common manifestation, caused by malformation of the joint with resultant instability and hip subluxation which leads to erosion of articular cartilage and synovitis (Zhu, Zhang et al. 2009). The lameness exhibited in this condition is caused by secondary osteoarthritis (Wood, Lakhani et al. 2004). Myopia has been linked to skeletal dysplasia in many human genetic syndromes including Stickler syndrome (Hakim, Elloumi et al. 2002), Kniest Syndrome (Mrugacz, Rydzanicz et al. 2009), and inherited hyaloideoretinopathy (Knobloch 1975), to name a few.

4.2. Aims of Chapter 4

4.2.1. Aim 4: To identify possible environmental factors which could contribute to refractive error in the Labrador Retriever

Following the findings of Aim One: that refractive error in the studied pedigree was inherited and that smaller litters demonstrated higher levels of myopia, further investigation of environmental influences was justified. Working with the same pedigree identified for Aim One, we gained access to breeding records which detailed litter size, date of birth, birthweight and information about post-natal health. All of this data was analysed in conjunction with adult refractive error to look for relationships between environmental variables.

Chapter Four outlines several environmental factors which may influence refractive error development in Labrador Retrievers. This chapter also follows on from chapter two as although familial aggregation analysis showed a genetic component, there was also a large amount of variance which was due to non-genetic factors i.e. environment. This again studied the colony of dogs used in chapter two. Because all of these dogs shared common environmental influences in the early stages of development, it provided a unique opportunity to study these factors. The major factors which were investigated both independently and in relation to adult refractive error were birth weight, growth rate, litter size, litter cohort and season of birth.

4.2.2. Specific Aims

1) To analyse the relationship between birthweight and Adult SER
2) To analyse the relationship between growth rate and Adult SER
3) To analyse the relationship between season of birth and Adult SER
4) To analyse the relationship between litter size and Adult SER
5) To analyse the relationship between season of birth and litter size

4.3. Methods

4.3.1. Litter Size

Mating, whelping and birth records were collected and collated. Breeds included were Labrador Retrievers, German Shepherds, Golden Retrievers, Curly Coated Retrievers, Poodles and Border Collies (and crosses between these breeds). Records were available describing pre-pregnancy health, mating records (ie. natural mating, slip mating, assisted, artificial insemination). Descriptive records were available for whelping including details of number of live births and any veterinary intervention such as caesarean section. These records were used to establish sizes of litters. Original breeding records were scanned and entered into an excel spreadsheet. Male and Female puppies were treated as two separate groups to compare gender effects. Only live births were included in litter numbers. Where puppies were stillborn or died before 6 weeks of age (died in nest) they were not included in litter size calculations.

4.3.2. Ocular and Systemic Pathology

Breeding records also described any significant ocular or systemic pathology. Disease prevalence rates were collated for each breed for the main conditions found across breeds. The main ocular pathologies were retinal folds; which are a sign of retinal dysplasia (discussed above) and cataracts. Less prevalent conditions were persistent papillary membrane or hyaloid remnants which are also a predictive sign of retinal dysplasia formation. Dogs that had any history of retinal folds were recorded as affected, even if the folds resolved following initial examination. The major systemic pathologies found across breeds were hip/elbow dysplasia, atopy, cancer and epilepsy. Skeletal dysplasia is particularly important as it has been associated with retinal dysplasia and progressive retinal atrophy (Carrig 1977) in Labrador Retrievers. Rates for each disease were calculated as prevalence rates across each breed and across the entire colony.

4.3.3. Birth weight and Growth Rate

Birth weight records were collated for all Labrador Retriever breeding females, but not for other breeds. Scanned records included birth weight tables with birth weights of each litter. All puppies were routinely weighed daily (by the breeding facility to ensure adequate daily

weight gain) from birth to at least four weeks of age, with some having weights up to six weeks of age. Where puppies were weighed more than once in a day the afternoon weight was used. Puppies were reportedly weighed at the same time of day, although the time of weighing was not recorded (except where puppies were weighed more than once per day where weights were recorded as AM and PM).

At the time of birth puppies are difficult to identify as they are born hairless and with their eyes closed. For that reason puppies had been marked with identifying dots on various locations (ear, head, back, tail etc). For the first six weeks puppies were identified by these markings and by their identification number rather than by name. The markings were matched to adult records to identify which dogs had been tested for refractive error.

An excel spreadsheet was constructed and daily weights were entered for all available litters of Labrador Retrievers. Daily and weekly weight gains were calculated for each puppy. These weights were collated into gender and litter size groups, which were categorised as small (1-4 puppies), medium (5-8 puppies) and large (9-13 puppies). Scatter plots were constructed to show growth rate vs litter size for the three groups for males and females.

Adult refractive error (SER) was graphed in relation to birth weight and weight at six weeks of age for males and females.

4.3.4. Early Environment

Pregnant bitches are brought to the Guide Dog Centre approximately 10 days before they are due. At this time they are isolated in a whelping room (Figure 14 (a)), with free access to water, and limited outdoor space. After whelping, puppies are confined to a small whelping box (Figure 14 (b)) until about two weeks of age, when they are allowed access to the larger room. Puppies are kept in this area until 6 weeks of age when they have their initial general vaccinations, health checks and ocular health examination. The rooms have natural light from windows (one wall only) all day and the puppies have limited exposure to outdoor space over three weeks of age. The rooms have fluorescent lighting which is switched on when needed (no regular cycle) and lights are switched off over night.

a

b

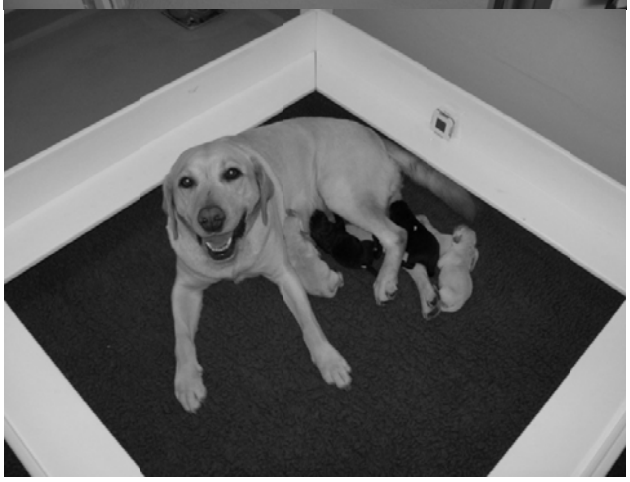


Figure 14 (a) Example of a room where puppies are kept isolated from birth until six weeks of age (b) nesting box where puppies are kept when whelped

4.3.5. Season of Birth

Season of birth was taken from whelping records using traditional Southern Hemisphere seasonal periods with spring running from September-November, summer from December-February, autumn from March-May and winter from June-August. Month of birth was available for all breeds, but refractive error comparisons were only available in Labrador Retrievers. For each season, average litter size, birth weight, growth rate, number of males vs females born and adult refractive error were analysed. As photoperiod has been implicated in previous studies involving human myopia development, average photoperiod was calculated for the day of birth for each dog and plotted versus adult SER. This was done by

calculating the difference between sunset and sunrise in Auckland which was available from the Royal Astronomical Society of New Zealand (www.rasnz.org.nz/SRSStimes.htm).

4.3.6. Statistical Analysis

Tests for normality were carried out for all continuous variables. Descriptive statistics were summarized using mean and standard deviation for parametric continuous variables, median and range for non-parametric continuous variables, and counts and percents for categorical variables. The maximum refractive error value in this population was +2.815 D, so to achieve an approximately normal distribution of refractive error, 2.815 was subtracted from each SER value before performing a cube-root transformation on the resulting values. A histogram plot of the distribution of refractive error showed that the data was left-skewed and had three outliers; the outliers were excluded from further statistical analysis.

Bivariate and multivariate analyses were performed using the MIXED procedure in SAS, with the identifier of Labradors from the same litter as the random effect. This accounted for clustering of refractive error measurements because there is a correlation of this data among Labradors from the same litter. In the bivariate analysis, we tested for independent associations between the transformed refractive error data and the following environmental factors: season of birth; litter size (tertiles); birth weight (tertiles); and average weight gain per week between birth and 4 weeks of age (tertiles). A similar analysis was done between transformed refractive error and the demographic factors, sex and age. Multivariate analysis was done between transformed refractive error and each environmental factor separately while adjusting for age and sex. We also tested for interaction effects between the environmental factor and demographic variables in the model. Results were summarized using means and adjusted means for bivariate and multivariate analysis, respectively. These were estimated by calculating the cube of the average transformed SER value from the model, and then subtracting the result from 2.815 (i.e. SER = 2.815 - [Transformed SER]^3). Statistical analysis was performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). As described above, different environmental variables had varying numbers of available data points, so the sample size differed for each variable.

A different statistical method was utilised in this chapter in comparison to chapter two even though some of the same data was used. Both sections used mixed model analysis, however chapter two studied refractive error in conjunction with the pedigree information of the larger family group. In this chapter, because a large group of animals was added to the analysis that

was unrelated to that studied in chapter two, litter number was entered as an identifier rather than entering the entire group as a pedigree (which would have led to a high proportion of founder individuals) . It should also be noted that environmental data was not available for all dogs included in the pedigree analysis in chapter two. The SAS software also allowed a greater number of variables to be compared, which was important for the environmental variables.

An overview of the environmental factors analysed and the sample sizes for each pair of factors in shown in Figure 15. The sample size is shown between each pair of factors.

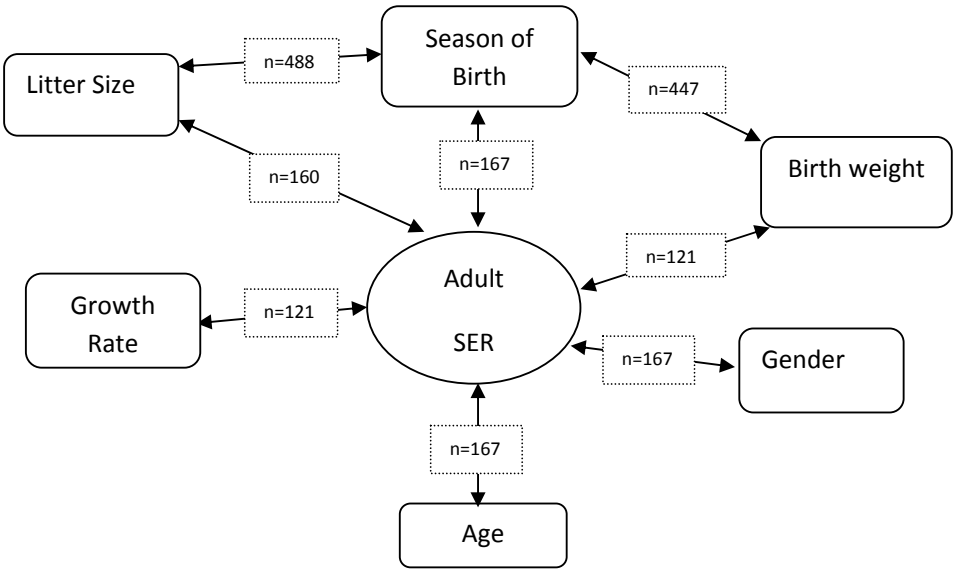


Figure 15 Diagram showing environmental factors analysed (for LR only). The sample size is shown between each pair of factors.

4.4. Results

4.4.1. Breeding Statistics

Table 9 summarises the breeding characteristics for each breed, including number of litters born (June 1986-June 2008), live births and stillbirths. Labrador retrievers represented the majority of dogs born within the colony (63%), followed by Labrador Retriever/Golden Retriever crosses (15%), Golden Retrievers (9%), German Shepherds (7%), Curly Coated Retrievers (and crosses) (3%), Border Collies (and crosses) (2%) and Poodles (1%).

Breed	Total # of Litters	Live Births number	Average Litter Size	SB (%)
LR	148	901	6.03	81
GR	19	128	6.74	12
GS	12	99	8.25	20
GRxLR	30	213	7.1	15
CCR/CCRx	6	35	5.83	0
BC/BCx	7	34	4.86	0
PDL	5	23	4.6	0
Total (Average)	**227**	**1433**	**(6.31)**	**128 (18.29%)**

Table 9 Summary of litter number for Labrador Retrievers (LR), Golden Retrievers (GR), German Shepherds (GS), Labrador Retriever/Golden Retrievers cross litters, Curly Coated Retrievers (CCR), and Curly Coated Retriever cross litters, Border Collie (BC) and Border Collie cross litters (BCx) and Poodles (PDL). Live births (at day of whelping), Average Litter Size (of live births) and Stillborn (SB) (at time of or on day of whelping)

4.4.2. Gestation

Gestation was taken from the day of 1st mating and was 60.4 days as an average across all breeds. There was no significant difference (1-way ANOVA, p=0.27) in length of gestation between breeds (range 59.56 (Labrador Retrievers) – 60.83 days (Border Collies/Border Collies crosses)). Of the 251 matings described across all of the breeds, 85% resulted in successful whelpings. The non-pregnancy rate of 15% across all breeds was the same in Labrador Retrievers only (n=162 matings).

4.4.3. Litter Size

Average litter size across all breeds was 6.31 puppies per litter (live births)(range 1-13), was slightly higher in Golden Retrievers (6.74), German Shepherds (8.25), Golden Retriever x Labrador Retriever (7.1) and lower in Labrador Retrievers (6.03), Curly Coated Retrievers (5.83), Border Collies (4.86) and Poodles (4.6). Litter size ranged from 1-13 puppies. There was no significant difference between litter number dependent on breed (p=0.39, 1-way ANOVA). Litter size was negatively correlated with length of gestation (r=-0.10, p=0.159).

The rate of stillbirths (SB) and deaths during the first week of life (in nest) varied between breeds, being much higher in some breeds. The highest rate of non-live births was in German Shepherds with 20%, followed by Golden Retrievers (9.3%), Labrador Retriever(8.9%) and Golden Retriever xLabrador Retriever (7%). No stillbirths were seen in Border Collies, Poodles or Curly Coated Retrievers but it should be noted that there were much smaller sample sizes in these breeds.

Multivariate statistical analysis using SAS showed no significant findings between litter size and adult refractive error (p=0.314), so did not replicate the finding in Chapter Two that dogs born into smaller litters (1-8) are significantly more myopic than those born into larger litters (9-13). However these conclusions were made with different sample sizes (116 (chapter two) vs 160 (current chapter)) and different statistical methods. Chapter two used a maximum likelihood variance component model whereas the current finding was made

using multivariate analysis.

Breeding females had an average of 2.5 litters (range 1-6) in their lifetime. The average age of whelping was 3.88 years of age with a range of 1.56-10.46 years of age. There was no relationship between age of whelping and adult refractive error (Pearson r= <0.001, p=0.854)).

4.4.4. Ocular/Systemic Pathology

Table 10 presents a summary of ocular and systemic pathology prevalence rates across all breeds. Retinal folds were the most common ocular pathology found in all breeds apart from border collies. Clinically significant retinal folds were seen in all other breeds with prevalence rates of up to 26% in the curly coated retriever (range 4.35-26%). Retinal folds were found both uni- (40%) and bilaterally (60%) and often resolved within 6 months of birth. The next most frequent finding was cataract, however this was found only in Labradors and Golden Retrievers and crosses of these breeds. Morphologically the cataracts were most frequently cortical in type, but anterior polar and nuclear sclerotic were also found. The most prevalent systemic pathology was hip/elbow dysplasia. The breed most commonly affected in our population was German Shepherds with a prevalence of 17.72% and the least frequently observed breed the Poodle at 4.35%. Atopy was not a prevalent pathology and only observed in a very small percentage of the population across all breeds at 0-2.34%.

Identification of a relationship between refractive error (SER) and ocular/systemic pathology was not directly possible, as all animals with ocular pathology were excluded from the study.

Breed	Congenital Cataracts	Retinal Folds	Hip/Elbow Dysplasia	Atopy
LR	2.55%	6.66%	8.32%	2.11%
GR	1.56%	7.81%	7.03%	2.34%
GS	0.00%	7.59%	17.72%	0.00%
GRxLR	2.35%	11.74%	9.39%	1.88%
CCR/CCRx	0.00%	25.71%	8.57%	0.00%
BC/BCx	0.00%	0.00%	5.88%	0.00%
PDL	0.00%	4.35%	4.35%	0.00%

Table 10 Summary of Ocular (congenital cataracts and retinal folds) and systemic pathology (Hip/Elbow Dysplasia and Atopy) findings across entire colony (n=1433). Prevalence is expressed as the

proportion of the breed affected by each condition including Labrador Retriever (LR), Golden Retriever (GR), German Shepherd (GS), Golden Retriever/Labrador Retriever cross (GRxLR), Curly Coated Retriever (CCR), and crosses (CCRx), Border Collie (BC) and crosses (BCx), and Poodle (PDL)

4.4.5. Birth weight and Growth Rate

Birth and growth rate data was collated for 457 Labradors from 70 litters produced by 27 brood bitches (litters born from 1995-2008). This subset of the Labrador population consisted of all available breeding records, being 82% of breeding females. Of the 457 puppies, 248 were male and 209 were female. Average litter size was 6.5 (range 1-13). Puppies were weighed daily from birth to between 33-56 days post-natal. A total of 20,108 weight observations were collated and analysed.

4.4.6. Birth Weight

Average birth weight overall was 463 ±52.9 grams, for males was 469 ±45 grams and for females 464 ±71grams, the difference not being significant (p=0.12). As demonstrated in Figure 16 average birth weights were lowest in puppies from large litters compared to medium and small litters. There was a significant difference found between small and medium litter size birth weight (BW) (p=0.01), and small and large litter size BW (p=0.003), but no significant difference was found between medium and large litter size BW (p=0.14). There was no significant difference in any of the litter size groups between average male and female BW (small p=0.72, medium p=0.15, large p=0.23 (t-test)).

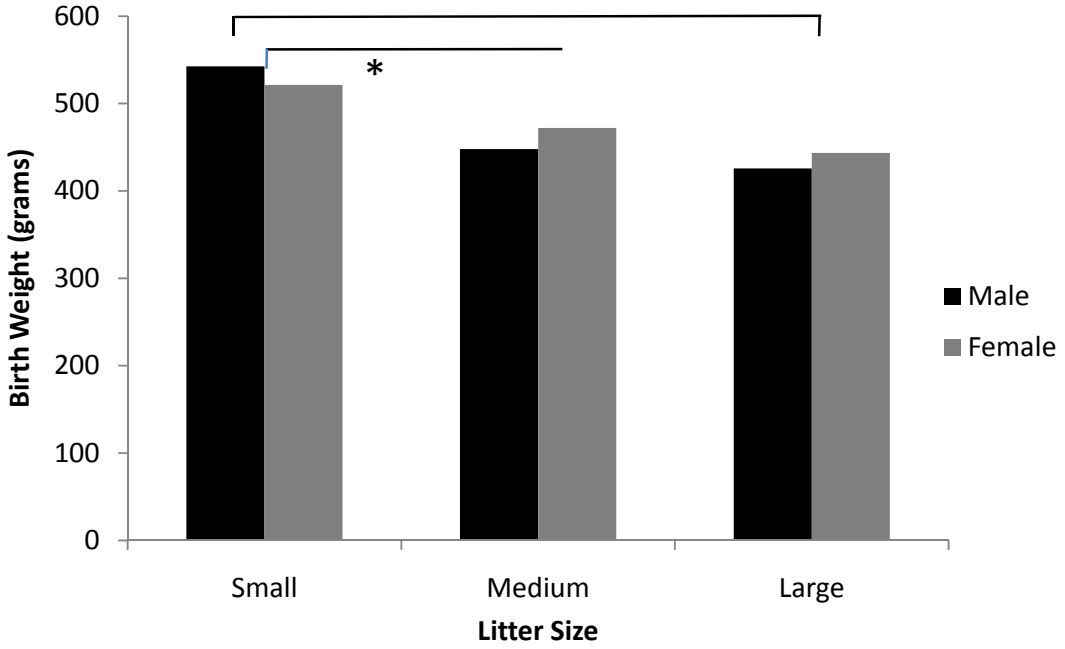


Figure 16 Graph showing birthweight (grams) of Labrador Retriever puppies (n=457). Small (S) litters include litters from 1-5 puppies, medium (M) from 6-8 and large (L) from 9-13. * indicates a significant result between groups ($p<0.05$)

4.4.7. Growth Rate

A similar trend continued with puppies from large litters showing the slowest growth rate and those from small litters showing the fastest growth rate (from birth to 4 weeks). Males showed a higher birth weight and faster growth rate across all three litter size groups, resulting in higher end weights at 35 days (Figure 17). A significant difference was found between small and medium ($p=0.008$(M), $p=0.006$(F)), medium and large ($p=0.005$(M), $p=0.03$(F)), and small and large litter growth rates ($p=<0.001$(M), $p=<0.001$(F)). A significant difference was found only between male and female growth rates in the small litter size group ($p=0.02$).

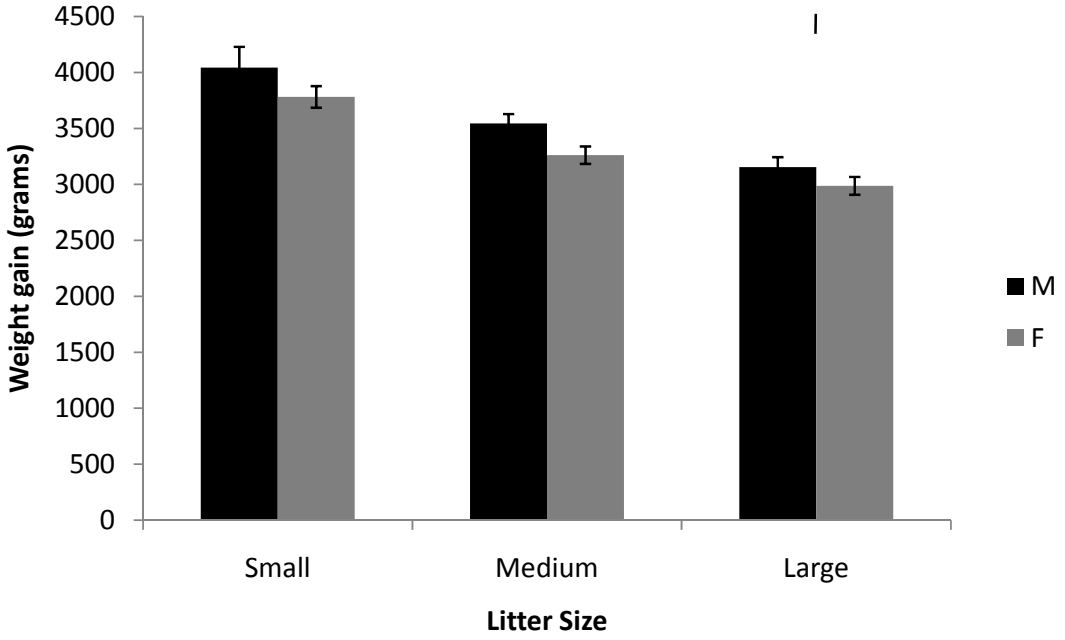


Figure 17 Graph showing rate of growth (weight gain (grams)) of Labrador Retriever puppies from 0-42 days after birth (n=457). Small (S) litters include litters from 1-5 puppies, medium (M) from 6-8 and large (L) from 9-13. * indicates a significant result between groups ($p<0.05$)

4.4.8. Adult SER and birth weight/growth rate

To identify any relationship between myopia and birth weight, male and female birth weights were plotted vs adult refractive error (SER) (Figure 18 (a) and (b)). The same graph was constructed with SER vs weight at 35 days (Figure 19 (a) and (b)). Analysis of these figures did not reveal a relationship between any of these variables with R^2 figures ranging from 0.0077 to 0.061 ($p=\geq 0.324$ for all correlations).

Bivariate and multivariate analysis showed no relationship between either birth weight or growth rate and adult SER. These results are detailed in Table 11 and Table 12.

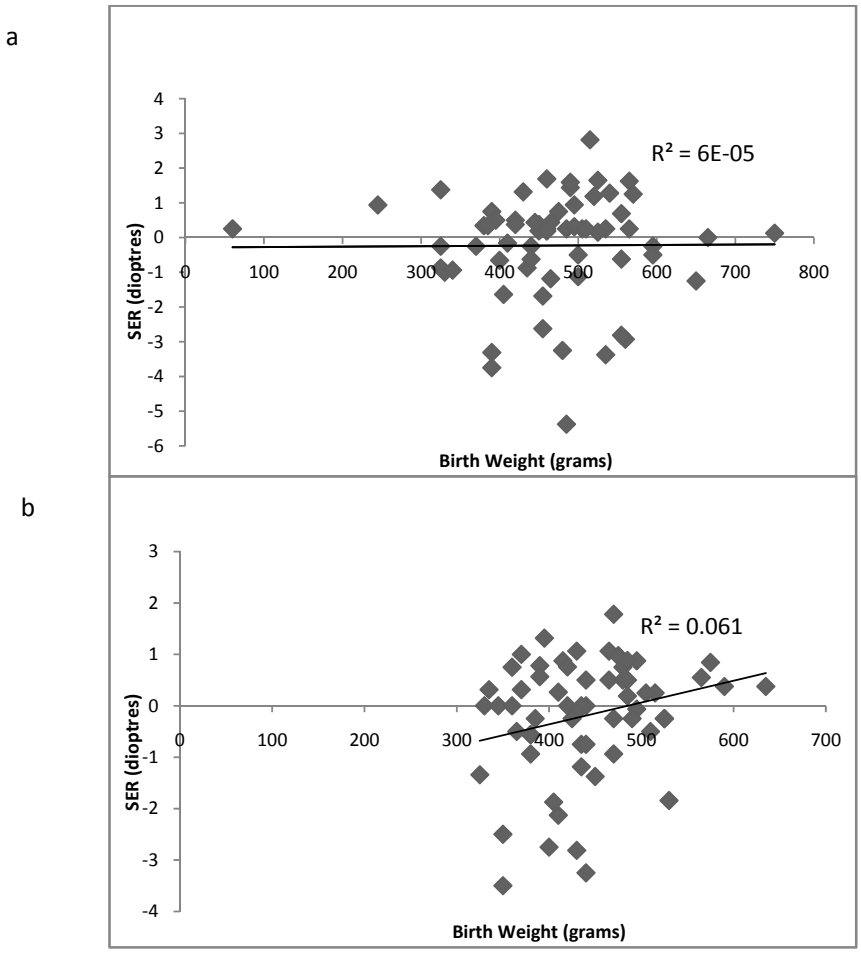
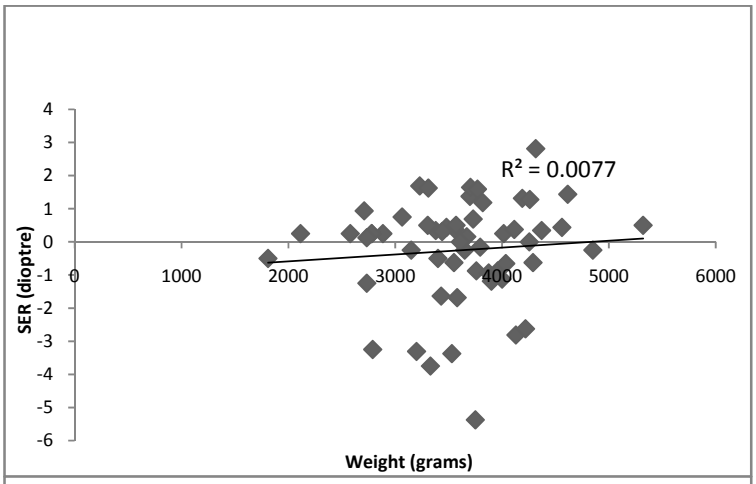


Figure 18 Scatter plots showing the relationship between birth weight and adult refractive errors for male (a) and female (b) Labrador Retrievers.

a



b

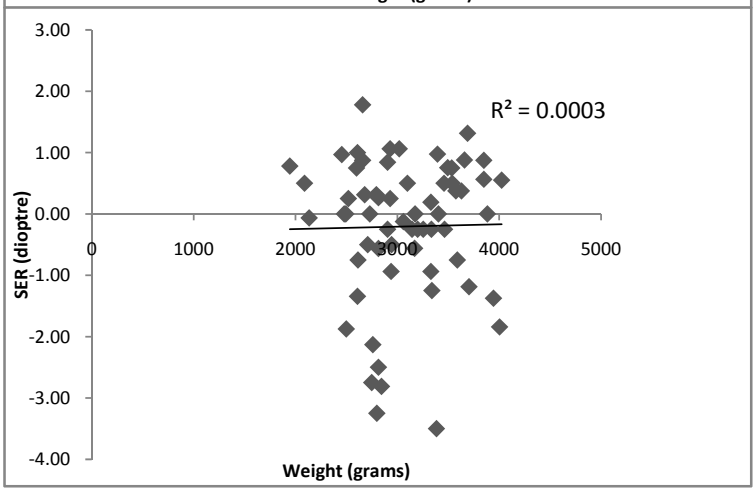


Figure 19 Scatter plot showing relationship between weight at 35 days of age vs adult refractive error for male (a) and female (b) Labrador Retriever's.

4.4.9. Season of Birth

The number of litters born per season was almost equally distributed (for all breeds). The most common seasons for birth were spring and winter (56 litters in each (26%)) followed by summer (52 litters (25%)) and autumn (48 litters (23%)), meaning that the most common seasons for conception were autumn and winter.

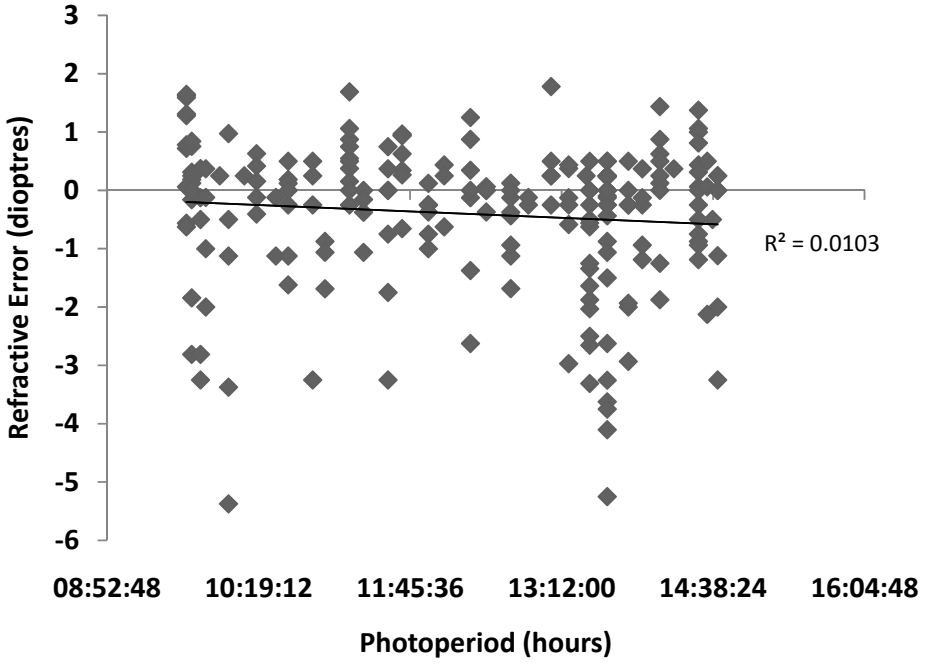


Figure 20 Scatter plot showing the relationship between photoperiod on day of birth and adult refractive error in Labrador Retriever's

Further analysis was undertaken for Labrador Retrievers regarding average photoperiod for date of birth. The average photoperiod on date of birth was 12:20:53 hours (range 09:38 - 14:41 hours). As shown in Figure 20 there is no apparent relationship between photoperiod and adult SER.

4.4.10. Season of Birth and Adult SER

It is clear from Figure 21 that there appears to be variation in the prevalence of refractive error by season, with more myopia apparent in spring and summer compared to autumn and winter. However, bivariate analysis showed no relationship between SER and season of birth (p=0.228, see table 11). Multivariate analysis adjusted for age and gender showed no significant effect in the upper and lower age tertiles. A significant result was found for the middle age tertile, with dogs between the ages of 17.23-29.67 months; born in spring or

summer being more myopic (p=0.023)(see Table 12). It should be noted that there was a large range in sample sizes, with autumn having only half the number of samples in comparison to summer and spring. It would be interesting to repeat this analysis with a larger more evenly distributed sample to increase power.

An association between litter size and season of birth was also tested for a larger sample size (n=488), showing a non-significant result (p=0.296). There was also no statistically significant difference between the birth weight of dogs born in different seasons (n=447 p=0.163).

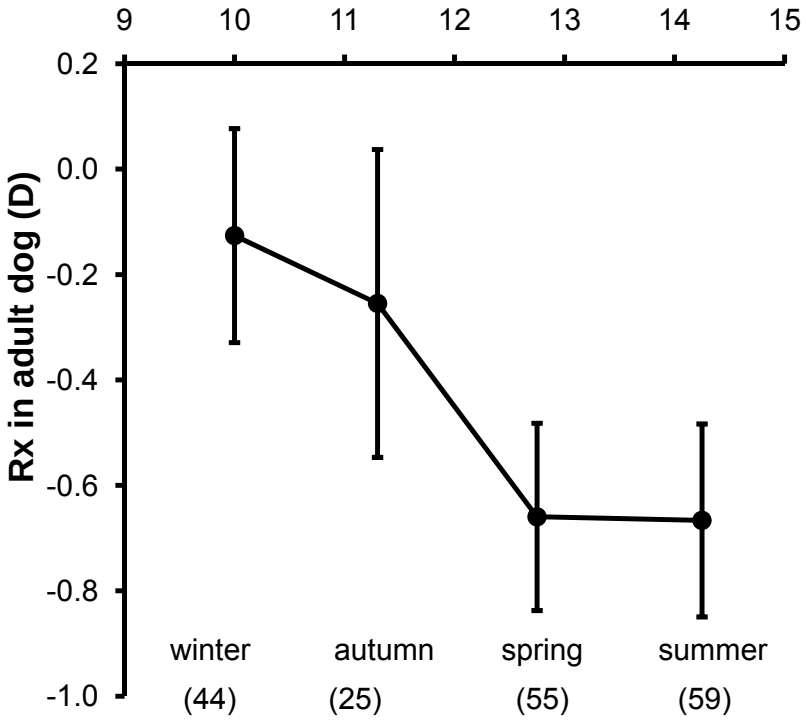


Figure 21 Graph showing relationship between season of birth and adult refractive error in Labrador Retriever's

When frequencies of refractive error groups are compared, spring and summer exhibit much greater proportions of myopia (43% and 33% respectively) compared to autumn and winter (24% and 28%). It is also of interest to note that of the 14 dogs who were classified as having hyperopia, 8 of these cases were born in winter (57%). This is demonstrated in

Figure 22. A chi-square analysis showed a non-significant result when refractive error was categorised as emmetropic, myopic or hyperopic and analysed against season of birth (categorised as spring-summer or autumn-winter, p=0.156).

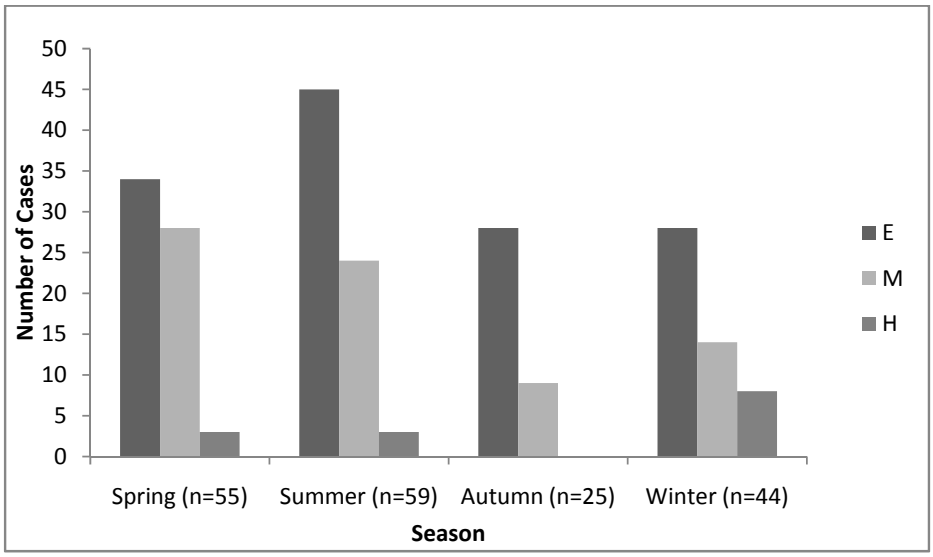


Figure 22 Bar plot showing season of birth in relation to proportion of refractive error categories – E (emmetropia), M (myopia), H (hyperopia)

4.4.11. Litter Parity

Litter parity refers to the litter order from a given breeding female. Brood bitches had between 1 and 5 litters. When refractive error from Labradors was averaged for each litter group a significant difference was found between groups (1-way ANOVA, p=<0.001). The most significant difference was found between average 5th litter SER compared to other groupings, although this represented the smallest dataset. This result may be due to a litter which showed a high level of myopia in the 5th litter group.

Litter parity was negatively correlated with litter size (r= -0.22, p=<0.01) across all breeds.

Table 11 Bivariate analysis between refractive error and environmental/demographic factors

Variable	SER Mean	p-value *
Season of birth (n=167) [a]		
Autumn	0.080	0.228
Spring	-0.438	
Summer	-0.456	
Winter	0.060	
Litter size (n=160) [a]		
1st tertile (1 to 6 litters)	-0.012	0.143
2nd tertile (7 to 8 litters)	0.020	
3rd tertile (9 to 12 litters)	-0.524	
Birth weight (grams) (n=121) [a]		
1st tertile (60.00 to 420.00 grams)	-0.382	0.322
2nd tertile (425.00 to 485.00 grams)	-0.039	
3rd tertile (490.00 to 750.00 grams)	0.023	
Average weight gain per week between birth and 4 weeks of age (grams) (n=121) [a]		
1st tertile (282.50 to 475.00 grams)	-0.053	0.835
2nd tertile (485.00 to 602.50 grams)	-0.211	
3rd tertile (605.00 to 991.25 grams)	-0.096	
Age (months) (n=167) [b]		
1st tertile (6.30 to 17.00 months)	-0.164	0.010
2nd tertile (17.23 to 29.67 months)	0.137	
3rd tertile (30.97 to 146.30 months)	-0.538	
Sex (n = 167)		
Female	-0.275	0.471
Male	-0.162	

* Overall p-value for the fixed effect (factor)
[a] No significant differences in SER shown in multiple comparison tests between variable strata
[b] The mean SER in the 2nd and 3rd age tertiles are significantly different (p=0.008)

Table 12 Multivariate analysis between refractive error and environmental factors adjusted for age and gender

Variable	SER Adjusted Mean	p-value *
Season of birth (n=170) [a, b]		
Autumn	0.069	0.409
Spring	-0.347	
Summer	-0.399	
Winter	0.066	
Litter size (n=160) [a]		
1st tertile (1 to 6 litters)	0.021	0.314
2nd tertile (7 to 8 litters)	-0.074	
3rd tertile (9 to 12 litters)	-0.432	
Birth weight (grams) (n=121) [a]		
1st tertile (60.00 to 420.00 grams)	-0.345	0.483
2nd tertile (425.00 to 485.00 grams)	-0.056	
3rd tertile (490.00 to 750.00 grams)	-0.033	
Average weight gain per week between birth and 4 weeks of age (grams) (n=121) [a]		
1st tertile (282.50 to 475.00 grams)	0.010	0.675
2nd tertile (485.00 to 602.50 grams)	-0.251	
3rd tertile (605.00 to 991.25 grams)	-0.156	

* Overall p-value for the fixed effect (factor)
[a] No significant differences in SER shown in multiple comparison tests between variable strata
[b] The relationship between SER and season differs by age tertile. There is a significant association between SER and season among Labradors in the 2nd age tertile (17-29 months) (p=0.023), but not in other age tertiles.

4.5. Discussion

Having evaluated a number of environmental factors in relation to adult refractive error in this Labrador Retriever pedigree, there do not seem to be any clear associations. This colony represented a unique opportunity for the study of environmental factors given the breeding history, thoroughness of records and definite genealogy. Having established that the population studied had a considerable level of myopia it was logical to proceed with an analysis of environmental variables which could influence refractive error development. This was particularly pertinent given the recent publication of literature surrounding environmental findings in children including natural light exposure and outdoor activity. The variables analysed are discussed in relation to adult refractive error as well as inter-variable interactions. Factors analysed in relation to adult SER were litter size, birth weight, growth rate, gender, season of birth and litter parity. Other variables analysed were the relationship between litter size and birth/growth rate, and these factors in relative to season of birth. The prevalence of ocular pathology is also discussed. Ocular pathology was not analysed relative to adult SER, as it was an exclusion criteria but was included as it is relevant to canine visual function. Ocular pathology may also contribute to the prevalence of ametropia, particularly if the condition was latent at the time of testing.

It is an remarkable evolutionary phenomenon that dogs have developed so many analogous human disease states since their domestication 15,000 years ago (Acland and Ostrander 2003). The cause of many of these conditions has been attributed to the intensive inbreeding of dogs, often originating from only a few founding individuals (Wayne and Ostrander 2004). However with a condition such as myopia, where there is clearly environmental influences the causation is likely multi-factorial, including genetic and environmental interactions. This is supported by the findings described in Chapter Two.

In comparison to their wild counterparts and ancestors, including the wolf; whose survival depends on continuous hunting and foraging, domestic dogs have almost no need to focus in the far distance. In fact, dogs raised in an urban domestic environment represent an animal

that is constrained to a very limited visual experience. Over many generations this may have created a tendency towards low to moderate levels of myopia, as there is no evolutionary benefit to having clear distance vision.

The above points do not apply to groups of dogs who are rated by their performance in distance visual tasks such as gun dogs who are required to spot an object falling at distances of several hundred metres. One would suspect that dogs involved in these sorts of tasks would have much lower levels of ametropia, in comparison to their domestic pet counterparts. One study supported this theory, finding that German Shepherds working as guide dogs had significantly lower levels of myopia compared to a matched pet control group (Murphy, Zadnik et al. 1992). Conversely it has also been shown that dogs with ametropia improve their sighting ability when fitted with corrective contact lenses, showing that refractive error does effect a dogs visual performance in tasks (Kubai, Bentley et al. 2008).

There is also the issue of genetic linkage where a desired physical characteristic is linked at a genetic level by a disease characteristic. This is seen in some lines of Dalmatians where particular coat colours are accompanied by unilateral deafness (Cargill, Famula et al. 2004).

The two populations of Labradors we have studied are interesting because they are bred for different purposes. The service colony which represents the majority of dogs tested, are bred predominantly for behavioural characteristics, to prevent ocular and systemic pathology and to some extent for overall physical traits such as weight and height. The other Labrador Retriever group tested are bred for breed-specific physical characteristics, as the success of the breeder is quantified by the number of wins their dogs' have in dog shows, and to a lesser extent for temperament, as the majority of puppies bred are sold as pets. Although the number of dogs phenotyped from breeders was smaller, the prevalence of myopia was much lower than in the service colony population (link to results chapter three). It may be that in the population of Labrador Retrievers bred for the service industry, where behavioural and health characteristics are more highly valued than physical traits, dogs that display desirable personality traits also have genetic loci which leave them susceptible to myopia development. The development of refractive error development/prevalence in this service colony is especially applicable to human myopia because their early environment more mimics that of a human child than regular pet or working dogs.

4.5.1. Birth-Rate and Litter Size

The finding from familial aggregation analysis in chapter two that litter size appeared to account for some of the variance in SER within the pedigree warranted more investigation into possible environmental influences. One possibility was that litter size would affect birth weight or growth weight in the early postnatal period, thus influencing eye growth and refractive error indirectly. We know from human studies that babies that are born preterm are more likely to have high refractive errors, so it seems in some ways counter intuitive that smaller litters would be more myopic. However, after re-analysing the data with a larger sample size and using a different statistical model, litter size was not shown to be associated with adult SER.

The average litter sizes found for different breeds were similar to those reported in other breeding colonies. Labrador Retrievers have been reported as having an average litter size of 8.2 puppies (Chatdarong, Tummaruk et al. 2007), in comparison to 6.03 puppies which was found in this colony.

The length of gestation has also been reported to differ between breeds, particularly between Labrador Retrievers and German Shepherds, with reports that Labrador Retrievers have larger litters than German Shepherds (Chatdarong, Tummaruk et al. 2007). The length of gestation has been shown to be related more to breed specific differences than to litter size (Okkens, Teunissen et al. 2001). No difference in litter size was found between breeds in the current study. However, this is a skewed result as Labrador Retrievers and crosses represented 78% of litter data. The whelping rate of 85% from matings is higher than that described in breed specific studies of Drever dogs (78.6%) (Gavrilovic, Andersson et al. 2008).

The rate of early canine mortality (including stillbirths and during the first seven weeks of life) has previously been reported as 22% in boxers (van der Beek, Nielen et al. 1999), and has been shown to be influenced by the rate of inbreeding, with increased mortality associated with higher inbreeding coefficients. Within litter effects also showed to be significant, due to exposure to infection during gestation, and genetic predispositions to still birth. The prevalence of early mortality observed in the GDS colony varied significantly depending on breed, but the level in Labrador Retrievers was slightly higher at 9%; than that previously reported. A small retrospective study reported the rate of early mortality (before 8 weeks of age) in Labrador Retrievers as 4% (Trangerud, Grondalen et al. 2007).

4.5.2. Birth Weight

Birth weight has previously been reported in several studies of Labrador Retrievers. The average birth weight found in this colony (for LR) for both male (469g) and female (464g) puppies was similar to that reported in other colonies (male=494g, female= 462g) (Trangerud, Grondalen et al. 2007), but showed less of a gender difference.

It was found, as described in other Labrador colonies (Helmink, Shanks et al. 2000) that birth rate was more dependent on litter size than on gender. Gender did not influence birth weight in any of the litter size categories, but there were significant differences seen between small and both medium and large litter size groups.

4.5.3. Ocular and Systemic Pathology

Although the rate of cataracts was reasonably low at 1.56-2.55%, all cases of cataract were detected in the first 18 months (geriatric cataracts were excluded). This makes canine cataracts clinically more analogous to congenital cataracts in babies which only occur in 2.49/10,000 (.025%) live births in the United Kingdom. Most cases of cataracts in the canine population were unilateral, whereas human congenital cataracts are more frequently bilateral (Rahi and Dezateux 2000).

The rate of retinal folds was high across breeds. Although this condition is not normally reported in German Shepherds as being heritable (Holle, Stankovics et al. 1999), this breed had the highest prevalence of retinal folds at 6 weeks of age. Retinal folds in German Shepherds have been previously reported in a guide dog colony (Holle, Stankovics et al. 1999). It is interesting to note that retinal folds reported at six weeks of age seemed to resolve in a large proportion of cases; when affected dogs were retested at six months of age. Retinal folds, when considered as a sign of retinal dysplasia are defined as a congenital abnormality of the retina (Acland and Aguirre 1995), which do not resolve with age. The observed resolution of retinal folds may be due to a different cause (other than retinal dysplasia), therefore being transitory in nature or represent a high rate of false positives during ophthalmological exam. This may be due to the young age at which the puppies are first tested; literature does suggest that it is difficult to accurately identify canine retinal folds under the age of 12 weeks (Holle, Stankovics et al. 1999). There is also evidence that the condition does not present in some animals until 10 weeks of age (Carrig, MacMillan et al.

1977). Perhaps the current ophthalmological health check regime used in the colony should be revised to recheck all dogs at 6 months of age.

4.5.4. Season of Birth

The suggestion that litters of one age group born in summer and spring demonstrated a higher level of myopia is interesting in relation to existing human research. The finding by Mandel et al that Israeli conscripts born in months with increased photoperiods concurred with our findings in Labrador Retrievers in the second age tertile, albeit in different months due to opposite hemispheres (Mandel, Grotto et al. 2008). It has been postulated in human studies that the effect of photoperiod on dopamine levels during pregnancy is one possible explanation for this pattern. Higher levels of dopamine in spring and summer occur due to more intense light exposure and longer photoperiods (Chotai, Joukamaa et al. 2009). As canine gestation is limited to 60 days there would not be such a great variation in light levels during gestation, as there would in humans.

There was no association found between season of birth and litter size. An association between with season of birth has been reported in German Shepherds born in Kenya, which found a difference in whelping rate (Mutembei, Mutiga et al. 2000) in different seasons but not in litter size (Mutembei, Mutiga et al. 2002). However, in other breeds of dog it has been shown that there is larger litters born in spring and autumn compared to winter and summer.

4.5.5. Litter Parity

A weak correlation was found between litter parity and adult refractive error. This data is by nature skewed, as there is much larger numbers within the first three litter groups, with fewer females having had 4^{th} or 5^{th} litters. There is little available data relating to canine disease and litter parity. However, there have been various reports about the effect of maternal age on various factors, which inherently relates to parity. Increased maternal age at whelping appears to decrease litter size, increase the rate of stillbirths and dystocia (difficult whelping) and increase the frequency of certain diseases in dogs. We also found a weak negative correlation between litter size and litter parity which has been previously described.

It is well established in human populations that increased maternal age increases the rate of miscarriage and stillbirth, and increases the risk of birth defects such as chromosomal abnormalities (Brown 2008). There is some evidence that older maternal age increases the

rate of myopia development in childhood (Rudnicka, Owen et al. 2008). There is also evidence that maternal age of >35years increases the rate of aniso-astigmatism and resultant amblyopia (Huynh, Wang et al. 2006).

4.5.6. Future Directions

Recently a number of studies (Jones, Sinnott et al. 2007; Rose, Morgan et al. 2008; Deere, Williams et al. 2009; Dirani, Tong et al. 2009) have investigated other possible environmental factors which could affect myopia development and progression. These studies have continued to look at near-work, but have also investigated time spent outdoors, and physical activity indoors and outdoors. The results from these studies convincingly show that time spent outdoors has a protective effect against myopia, although the exact protective factor is difficult to isolate. One study has also shown that physical activity levels are also decreased in myopic children, regardless of whether in/outdoors, but did not comment on light exposure (Deere, Williams et al. 2009). It would be interesting to evaluate these factors in this colony of dogs, and to conduct studies during development with different levels/durations of natural light exposure. To further develop the model, it would also be of value to study the normal refractive development of Labrador Retriever puppies over the first six months of life.

Further study in this pedigree would be valuable to phenotype a greater number of generations of Labradors for refractive error. An increase in sample size would enable more accurate estimates of seasonal and developmental effects on resultant adult refractive error. It would also be interesting to collate similar data on other breeds of dog, particularly those which have been identified as having a high prevalence of myopia (such as Rottweilers and Schnauzers) (Murphy, Zadnik et al. 1992). Measures of other potential environmental influences on myopia development such as levels of light exposure, levels of physical activity and time indoors/outdoors would be informative. In dogs there is the additional advantage to be able to control these parameters to monitor the effect on refractive error.

There is not much literature available surrounding canine visual function. It would be interesting to assess breed specific visual functions. As skull shape and size influences eye position and visual streak morphology, general values for canine visual characteristics may be misleading. It would be interesting to further investigate canine visual functions including binocular vision parameters such as stereopsis, phorias, convergence and fusional ranges. Re-examining the amplitude of accommodation would also be informative as it is frequently

quoted as being low, in the range of 1-2 dioptres in dogs. However, the original reference for this dates back to Hess et al (Hess 1898) where stimulation of the sympathetic nerve elicited this response. Testing accommodative amplitude and near visual capacity using behavioural methods would give more functional values.

Chapter 5. General Conclusions

5.1. Familial Aggregation Analysis

The familial aggregation analysis described in chapter two demonstrates that the dog is the first non-human species to show refractive error with an inherited component. The dog has also demonstrated naturally occurring myopia in a number of studies, which has not been shown consistently in any other animal species (Kubai, Bentley et al. 2008). The currently studied pedigree of Labrador Retrievers has shown a higher prevalence of myopia at 37% than previously reported. There was also a greater range of refractive error (-5.62 to +2.82) than previously reported in this breed.

After trialling several methods of testing canine refractive error, cycloplegic retinoscopy offers the most reliable, portable, and flexible solution, as has been found in many other studies of refractive error in animals.

The statistical analysis was completed with software that was able to take into account all familial relationships within the family, which is important when working with pedigrees with a high rate of consanguinity. It was shown that there was a significant portion of variance due to genetic effects; suggesting a heritable component to refractive error development in this pedigree. Dogs from small litters were shown to be significantly more myopic than dogs from larger litters. The almost equal amount of variance due to non-genetic effects warranted more study into possible environmental influences, which is further discussed in Chapter Two.

In summary the main findings from Chapter two were that within the pedigree studied the Labrador Retriever demonstrated a high prevalence of naturally occurring myopia, which was shown to have a significant heritable component.

5.2. Genetic Analysis

The dog has many advantages for the genetic study of human disease as many of the same conditions occur naturally in the dog with the same underlying physiological mechanisms. As described in chapter three, DNA samples are easily collected from dogs in the form of buccal swabs or saliva samples. Chapter three also demonstrates that relationship parameters

required for genetic studies are easily obtained and calculated due to the known relationships and pedigree records available in pedigree dogs. Due to the fully sequenced dog genome and increasing technologies available in canine genetics including whole genome SNP arrays, these studies are becoming more accessible. The dog provides a valuable model for the genetic study of myopia, as both the human and dog genomes become more finely sequenced there will be greater opportunities for the discovery of shared disease causing sequences.

Although the SNP genotyping was not completed due to a high rate of amplification failure, all methods are now in place to complete this line of study. As discussed in Chapter two, given the highly inbred nature of pedigree animals, particular attention has to be given to using software which is able to account for these relationships. Using relationship coefficients and pedigree files, it was demonstrated that there is a level of relatedness between most Labradors in New Zealand. If taken into consideration when designing a genotyping study these relationships can make gene discovery more achievable.

5.3. Environmental Factors

One of the major advantages of the dog as an animal model of naturally occurring myopia is the ability to alter environmental influences to study the effect on refractive development.

Chapter four established that a number of factors had an influence on refractive error including age, litter parity and season of birth (in one age tertile). Factors which did not seem to have an effect on adult refractive error were litter size, birth weight and growth rate.

There is a great amount of knowledge which could be gained by further exploring environmental factors during canine development. Little is known about the refractive and ocular development of puppies over the first few months of life. Altering light exposure and the visual environment may have a greater influence on adult refractive error in dogs.

This section also showed that dogs have a range of ocular and systemic pathologies which are analogous to human conditions.

In conclusion, this thesis has demonstrated that the Labrador Retriever represents a valuable and unique animal model for the study of naturally occurring, heritable myopia.

References

Acland, G. M. and G. D. Aguirre (1995). Oculoskeletal dysplasias in Samoyed and Labrador Retriever dogs: nonallelic disorders akin to Stickler-like syndromes affecting humans. 2nd International DOGMAP Meeting, Cambridge, UK.

Acland, G. M. and E. A. Ostrander (2003). "The dog that came in from the cold." Heredity **90**(3): 201-2.

Acland, G. M., K. Ray, et al. (1998). "Linkage analysis and comparative mapping of canine progressive rod-cone degeneration (prcd) establishes potential locus homology with retinitis pigmentosa (RP17) in humans." Proc Natl Acad Sci U S A **95**(6): 3048-53.

Angi, M. R., M. Clementi, et al. (1993). "Heritability of myopic refractive errors in identical and fraternal twins." Graefes Arch Clin Exp Ophthalmol **231**(10): 580-5.

Arcos-Burgos, M. and M. Muenke (2002). "Genetics of population isolates." Clin Genet **61**(4): 233-47.

Ashton, G. C. (1985). "Segregation analysis of ocular refraction and myopia." Hum Hered **35**(4): 232-9.

Avila, M. P., J. J. Weiter, et al. (1984). "Natural history of choroidal neovascularization in degenerative myopia." Ophthalmology **91**(12): 1573-81.

Awano, T., G. S. Johnson, et al. (2009). "Genome-wide association analysis reveals a SOD1 mutation in canine degenerative myelopathy that resembles amyotrophic lateral sclerosis." Proc Natl Acad Sci U S A **106**(8): 2794-9.

Bagchi, S. and H. M. Salihu (2006). "Birth weight discordance in multiple gestations: occurrence and outcomes." J Obstet Gynaecol **26**(4): 291-6.

Bamashmus, M. A., B. Matlhaga, et al. (2004). "Causes of blindness and visual impairment in the West of Scotland." Eye **18**(3): 257-61.

Bardien, S., R. Ramesar, et al. (1997). "Retinitis pigmentosa locus on 17q (RP17): fine localization to 17q22 and exclusion of the PDEG and TIMP2 genes." Hum Genet **101**(1): 13-7.

Bartsocas, C. S. and A. D. Kastrantas (1981). "X-linked form of myopia." Hum Hered **31**(3): 199-200.

Bayes, M., A. Martinez-Mir, et al. (1996). "Autosomal recessive retinitis pigmentosa in Spain: evaluation of four genes and two loci involved in the disease." Clin Genet **50**(5): 380-7.

Belkin, M., U. Yinon, et al. (1977). "Effect of visual environment on refractive error of cats." Doc Ophthalmol **42**(2): 433-7.

Bellis, M. A., K. Hughes, et al. (2005). "Measuring paternal discrepancy and its public health consequences." J Epidemiol Community Health **59**(9): 749-54.

Biino, G., M. A. Palmas, et al. (2005). "Ocular refraction: heritability and genome-wide search for eye morphometry traits in an isolated Sardinian population." Hum Genet **116**(3): 152-9.

Black, J. C., A. Phillips, J. Souhkhaphanh, J (2004). The contrast sensitvity function of the dog, University of Auckland

Black, J. M., S. R. Browning, et al. (2008). "A canine model of inherited myopia: Familial aggregation of refractive error in Labrador Retrievers." Invest Ophthalmol Vis Sci.

Bodnar, L. M. and H. N. Simhan (2008). "The prevalence of preterm birth and season of conception." Paediatr Perinat Epidemiol **22**(6): 538-45.

Borecki, I. B. and B. K. Suarez (2001). "Linkage and association: basic concepts." Adv Genet **42**: 45-66.
Borthakur, G., C. Butryee, et al. (2008). "Exfoliated buccal mucosa cells as a source of DNA to study oxidative stress." Cancer Epidemiol Biomarkers Prev **17**(1): 212-9.
Bouchard, G., R. S. Youngquist, et al. (1991). "Seasonality and variability of the interestrous interval in the bitch." Theriogenology **36**(1): 41-50.
Bradley, D. V., A. Fernandes, et al. (1999). "The refractive development of untreated eyes of rhesus monkeys varies according to the treatment received by their fellow eyes." Vision Res **39**(10): 1749-57.
Brandies, R. and S. Yehuda (2008). "The possible role of retinal dopaminergic system in visual performance." Neurosci Biobehav Rev **32**(4): 611-56.
Bromberg, N. M. D., W.W. (1980). "Preliminary measures of canine visual spatial resolution with electrophysiological techniques. ." Trans. Am. Coll. Vet. Ophthalmol. **11**: 120-125.
Brown, S. (2008). "Miscarriage and its associations." Semin Reprod Med **26**(5): 391-400.
Bullimore, M. A., L. A. Jones, et al. (2002). "A retrospective study of myopia progression in adult contact lens wearers." Invest Ophthalmol Vis Sci **43**(7): 2110-3.
Cadieu, E., M. W. Neff, et al. (2009). "Coat variation in the domestic dog is governed by variants in three genes." Science **326**(5949): 150-3.
Cagigrigoriu, A., D. Gregori, et al. (2007). "Heritability of corneal curvature and astigmatism: a videokeratographic child-parent comparison study." Cornea **26**(8): 907-12.
Cargill, E. J., L. A. Clark, et al. (2002). "Multiplexing of canine microsatellite markers for whole-genome screens." Genomics **80**(3): 250-3.
Cargill, E. J., T. R. Famula, et al. (2004). "Heritability and segregation analysis of deafness in U.S. Dalmatians." Genetics **166**(3): 1385-93.
Carrig, C. B., A. MacMillan, et al. (1977). "Retinal dysplasia associated with skeletal abnormalities in Labrador Retrievers." J Am Vet Med Assoc **170**(1): 49-57.
Carter, S. D., A. Barnes, et al. (1999). "Canine rheumatoid arthritis and inflammatory cytokines." Vet Immunol Immunopathol **69**(2-4): 201-14.
Chang, M. L., R. L. Terrill, et al. (2007). "Large-scale SNP genotyping with canine buccal swab DNA." J Hered **98**(5): 428-37.
Chapell, M., B. Sullivan, et al. (2001). "Myopia and night-time lighting during sleep in children and adults." Percept Mot Skills **92**(3 Pt 1): 640-2.
Chatdarong, K., P. Tummaruk, et al. (2007). "Seasonal and breed effects on reproductive parameters in bitches in the tropics: a retrospective study." J Small Anim Pract **48**(8): 444-8.
Chawla, S. K. and J. F. Reece (2002). "Timing of oestrus and reproductive behaviour in Indian street dogs." Vet Rec **150**(14): 450-1.
Chen, C. T., K. Alyahya, et al. (2008). "Loss of glutamine synthetase immunoreactivity from the retina in canine primary glaucoma." Vet Ophthalmol **11**(3): 150-7.
Chen, C. Y., K. J. Scurrah, et al. (2007). "Heritability and shared environment estimates for myopia and associated ocular biometric traits: the Genes in Myopia (GEM) family study." Hum Genet **121**(3-4): 511-20.
Chen, C. Y., J. Stankovich, et al. (2007). "Linkage replication of the MYP12 locus in common myopia." Invest Ophthalmol Vis Sci **48**(10): 4433-9.
Chew, S. J. and V. Balakrishnan (1992). "Myopia produced in young chicks by intermittent minimal form visual deprivation--can spectacles cause myopia?" Singapore Med J **33**(5): 489-92.

Choi, M. Y., I. K. Park, et al. (2000). "Long term refractive outcome in eyes of preterm infants with and without retinopathy of prematurity: comparison of keratometric value, axial length, anterior chamber depth, and lens thickness." Br J Ophthalmol **84**(2): 138-43.

Chotai, J. and R. Adolfsson (2002). "Converging evidence suggests that monoamine neurotransmitter turnover in human adults is associated with their season of birth." Eur Arch Psychiatry Clin Neurosci **252**(3): 130-4.

Chotai, J., M. Joukamaa, et al. (2009). "Novelty seeking among adult women is lower for the winter borns compared to the summer borns: replication in a large Finnish birth cohort." Compr Psychiatry **50**(6): 562-6.

Chougnet, C., P. Deloron, et al. (1990). "Humoral and cell-mediated immune responses to the Plasmodium falciparum antigens PF155/RESA and CS protein: seasonal variations in a population recently reexposed to endemic malaria." Am J Trop Med Hyg **43**(3): 234-42.

Christie, D. W. and E. T. Bell (1971). "Endocrinology of the oestros cycle in the bitch." J Small Anim Pract **12**(7): 383-9.

Coile, D. C., C. H. Pollitz, et al. (1989). "Behavioral determination of critical flicker fusion in dogs." Physiol Behav **45**(6): 1087-92.

Collins, A. (2004). Visual acuity and guiding performance in a group of guide dogs: Effect of naturally-occurring myopia. 10th International myopia conference, Cambridge.

Cooperstock, M. and R. A. Wolfe (1986). "Seasonality of preterm birth in the Collaborative Perinatal Project: demographic factors." Am J Epidemiol **124**(2): 234-41.

Corbett, R. (2002). Familial Trends of Refractive Error, Retinoscopy and Photoretinoscopy in a Population of Guide Dogs. Department of Optometry and Vision Science. Auckland, University of Auckland**:** 33.

Corbett, R. D., C. Collins, AV. Phillips, JR. (2002). Familial trends in refractive error in a group of guide dogs. 9th International Myopia conference., Hong Kong.

Cordonnier, M. and V. De Maertelaer (2004). "Comparison between two hand-held autorefractors: the Sure-Sight and the Retinomax." Strabismus **12**(4): 261-74.

Cosgrave, E., C. Scott, et al. (2008). "Ocular findings in low birthweight and premature babies in the first year: do we need to screen?" Eur J Ophthalmol **18**(1): 104-11.

Cui, X., A. McGregor, et al. (2008). "Cloning the complete guinea pig cytomegalovirus genome as an infectious bacterial artificial chromosome with excisable origin of replication." J Virol Methods **149**(2): 231-9.

Curtin, B. J. (1985). The myopias : basic science and clinical management. Philadelphia, Harper & Row.

Curtis, R. B., KC. (1989). "A survey of cataracts in Golden and Labrador Retrievers. ." J Small Anim Pract **30**(30): 277-286.

Dassa, D. and J. M. Azorin (1993). "Season of birth and bipolar disorder." Am J Psychiatry **150**(3): 526-7.

Davidson, M. G., C. J. Murphy, et al. (1993). "Refractive state of aphakic and pseudophakic eyes of dogs." Am J Vet Res **54**(1): 174-7.

Davis, G. E., Jr. and W. E. Lowell (2006). "Solar cycles and their relationship to human disease and adaptability." Med Hypotheses **67**(3): 447-61.

Davitt, B. V., V. Dobson, et al. (2005). "Prevalence of myopia at 9 months in infants with high-risk prethreshold retinopathy of prematurity." Ophthalmology **112**(9): 1564-8.

De Jong, P. T., B. A. Oostra, et al. (1993). "High symmetric anisometropia in monozygotic twins." Ophthalmic Paediatr Genet **14**(1): 29-32.

Deere, K., C. Williams, et al. (2009). "Myopia and later physical activity in adolescence: a prospective study." Br J Sports Med **43**(7): 542-4.

Dirani, M., M. Chamberlain, et al. (2006). "Refractive errors in twin studies." Twin Res Hum Genet **9**(4): 566-72.
Dirani, M., M. Chamberlain, et al. (2006). "Heritability of refractive error and ocular biometrics: the Genes in Myopia (GEM) twin study." Invest Ophthalmol Vis Sci **47**(11): 4756-61.
Dirani, M., A. Islam, et al. (2008). "Dominant genetic effects on corneal astigmatism: the genes in myopia (GEM) twin study." Invest Ophthalmol Vis Sci **49**(4): 1339-44.
Dirani, M., S. N. Shekar, et al. (2008). "The role of educational attainment in refraction: the Genes in Myopia (GEM) twin study." Invest Ophthalmol Vis Sci **49**(2): 534-8.
Dirani, M., L. Tong, et al. (2009). "Outdoor activity and myopia in Singapore teenage children." Br J Ophthalmol **93**(8): 997-1000.
DNAgenotek (2008) "Laboratory Protocol for Manual Purification of DNA from 0.5mL of Oragene DNA/Saliva." **Volume**, DOI:
Donaghy, M. (1980). "The cat's vestibulo-ocular reflex." J Physiol **300**: 337-51.
Dubar, M. J. T., M.G. (1920). "Essai de determination de la refraction statique de loeil du chien et du chat." Comptes Rendus Hebdomadores des Seances. Academie de Sciences **183**: 912-914.
Duke-Elder, S. (1958). The eye in evolution, Kimpton.
Duke-Elder, S. A., D. (1970). System of Optthalmology, C.V. Mosby.
Edwards, M. H. (1998). "Effect of parental myopia on the development of myopia in Hong Kong Chinese." Ophthalmic Physiol Opt **18**(6): 477-83.
Egashira, S. M., L. L. Kish, et al. (1993). "Comparison of cyclopentolate versus tropicamide cycloplegia in children." Optom Vis Sci **70**(12): 1019-26.
Eggleston, M. L., D. N. Irion, et al. (2002). "PCR multiplexed microsatellite panels to expedite canine genetic disease linkage analysis." Anim Biotechnol **13**(2): 223-35.
Eilts, B. E., A. P. Davidson, et al. (2005). "Factors affecting gestation duration in the bitch." Theriogenology **64**(2): 242-51.
Elston, R. C. (1981). "Segregation analysis." Adv Hum Genet **11**: 63-120, 372-3.
Elston, R. C. (1998). "Linkage and association." Genet Epidemiol **15**(6): 565-76.
Elston, R. C. (2000). "Introduction and overview. Statistical methods in genetic epidemiology." Stat Methods Med Res **9**(6): 527-41.
Farbrother, J. E., G. Kirov, et al. (2004). "Family aggregation of high myopia: estimation of the sibling recurrence risk ratio." Invest Ophthalmol Vis Sci **45**(9): 2873-8.
Feldkamper, M. and F. Schaeffel (2003). "Interactions of genes and environment in myopia." Dev Ophthalmol **37**: 34-49.
Feldman, E. C. and R. W. Nelson (2004). Canine and feline endocrinology and reproduction. St Louis, Missouri. , Saunders.
Fernando, O., P. Mota, et al. (2005). "Peopling of the Azores Islands (Portugal): data from the Y chromosome." Hum Biol **77**(2): 189-99.
Fisher, R. A. (1918). "The correlation between relatives on the supposition of Mendelian inheritance. ." Trans. Roy. Soc. Edin. **52**: 399-433.
Fisher, R. A. (1958). The Genetical Theory of Natural Selection. . New York. , Dover
Fotedar, R., P. Mitchell, et al. (2008). "Relationship of 10-year change in refraction to nuclear cataract and axial length findings from an older population." Ophthalmology **115**(8): 1273-8, 1278 e1.
Francisco, L. V., A. A. Langston, et al. (1996). "A class of highly polymorphic tetranucleotide repeats for canine genetic mapping." Mamm Genome **7**(5): 359-62.
Fredrick, D. R. (2002). "Myopia." BMJ **324**(7347): 1195-9.

Gao, Q., Q. Liu, et al. (2006). "Effects of direct intravitreal dopamine injections on the development of lid-suture induced myopia in rabbits." Graefes Arch Clin Exp Ophthalmol **244**(10): 1329-35.
Garner, L. F., C. K. Meng, et al. (1990). "Ocular dimensions and refractive power in Malay and Melanesian children." Ophthalmic Physiol Opt **10**(3): 234-8.
Garner, L. F., H. Owens, et al. (1999). "Prevalence of myopia in Sherpa and Tibetan children in Nepal." Optom Vis Sci **76**(5): 282-5.
Garner, L. F., A. W. Stewart, et al. (2004). "The Nepal longitudinal study: predicting myopia from the rate of increase in vitreous chamber depth." Optom Vis Sci **81**(1): 44-8.
Garoufalis, P., C. Y. Chen, et al. (2007). "Evaluation of accuracy in proband-reported family history and its determinants: the Genes in Myopia family study." Optom Vis Sci **84**(6): 481-6.
Gavrilovic, B. B., K. Andersson, et al. (2008). "Reproductive patterns in the domestic dog--a retrospective study of the Drever breed." Theriogenology **70**(5): 783-94.
Genin, E. and F. Clerget-Darpoux (1996). "Association studies in consanguineous populations." Am J Hum Genet **58**(4): 861-6.
Glickstein, M. and M. Millodot (1970). "Retinoscopy and eye size." Science **168**(931): 605-6.
Goodchild, A. K., K. K. Ghosh, et al. (1996). "Comparison of photoreceptor spatial density and ganglion cell morphology in the retina of human, macaque monkey, cat, and the marmoset Callithrix jacchus." J Comp Neurol **366**(1): 55-75.
Gorig, C. (2000). Ocular biometry and ophthalmological examination of canine breeds predisposed to cataracts and dogs with cataracts. School of Veterinary Medicine. Hannover, University of Hannover. **Tierarztliche Hochschule Dissertation**.
Goss, D. A. and T. W. Jackson (1996). "Clinical findings before the onset of myopia in youth: 4. Parental history of myopia." Optom Vis Sci **73**(4): 279-82.
Graham, B. and S. J. Judge (1999). "The effects of spectacle wear in infancy on eye growth and refractive error in the marmoset (Callithrix jacchus)." Vision Res **39**(2): 189-206.
Graham, B. and S. J. Judge (1999). "Normal development of refractive state and ocular component dimensions in the marmoset (Callithrix jacchus)." Vision Res **39**(2): 177-87.
Griebel, U. and A. Schmid (1997). "Brightness discrimination ability in the West Indian manatee (Trichechus manatus)." J Exp Biol **200**(Pt 11): 1587-92.
Grjibovski, A. M., P. Magnus, et al. (2006). "Epidemiology and heritability of astigmatism in Norwegian twins: an analysis of self-reported data." Ophthalmic Epidemiol **13**(4): 245-52.
Grosvenor, T. (2003). "Why is there an epidemic of myopia?" Clin Exp Optom **86**(5): 273-5.
Grosvenor, T. and D. A. Goss (1998). "Role of the cornea in emmetropia and myopia." Optom Vis Sci **75**(2): 132-45.
Guenet, J. L. (2005). "The mouse genome." Genome Res **15**(12): 1729-40.
Guggenheim, J. A., C. Hill, et al. (2003). "Myopia, genetics, and ambient lighting at night in a UK sample." Br J Ophthalmol **87**(5): 580-2.
Guggenheim, J. A., G. Kirov, et al. (2000). "The heritability of high myopia: a reanalysis of Goldschmidt's data." J Med Genet **37**(3): 227-31.
Guggenheim, J. A., R. Pong-Wong, et al. (2007). "Correlations in refractive errors between siblings in the Singapore Cohort Study of Risk factors for Myopia." Br J Ophthalmol **91**(6): 781-4.
Guo, S. S., J. G. Sivak, et al. (1996). "Effects of continuous light on experimental refractive errors in chicks." Ophthalmic Physiol Opt **16**(6): 486-90.
Guyon, R., T. D. Lorentzen, et al. (2003). "A 1-Mb resolution radiation hybrid map of the canine genome." Proc Natl Acad Sci U S A **100**(9): 5296-301.

Guzowski, M., J. J. Wang, et al. (2003). "Five-year refractive changes in an older population: the Blue Mountains Eye Study." Ophthalmology **110**(7): 1364-70.
Gwiazda, J., E. Ong, et al. (2000). "Myopia and ambient night-time lighting." Nature **404**(6774): 144.
Gwiazda, J., F. Thorn, et al. (1993). "Myopic children show insufficient accommodative response to blur." Invest Ophthalmol Vis Sci **34**(3): 690-4.
Haim, M., H. C. Fledelius, et al. (1988). "X-linked myopia in Danish family." Acta Ophthalmol (Copenh) **66**(4): 450-6.
Hakim, H., M. Elloumi, et al. (2002). "[Polyarthritic manifestations revealing Stickler syndrome]." J Radiol **83**(12 Pt 1): 1856-8.
Hammond, C. J., T. Andrew, et al. (2004). "A susceptibility locus for myopia in the normal population is linked to the PAX6 gene region on chromosome 11: a genomewide scan of dizygotic twins." Am J Hum Genet **75**(2): 294-304.
Hammond, C. J., H. Snieder, et al. (2001). "Genes and environment in refractive error: the twin eye study." Invest Ophthalmol Vis Sci **42**(6): 1232-6.
Han, W., K. H. Leung, et al. (2009). "Association of PAX6 polymorphisms with high myopia in Han Chinese nuclear families." Invest Ophthalmol Vis Sci **50**(1): 47-56.
Hayes, H. M., Jr., G. P. Wilson, et al. (1982). "Carcinoma of the nasal cavity and paranasal sinuses in dogs: descriptive epidemiology." Cornell Vet **72**(2): 168-79.
He, M., D. Wang, et al. (2008). "Heritability of anterior chamber depth as an intermediate phenotype of angle-closure in Chinese: the Guangzhou Twin Eye Study." Invest Ophthalmol Vis Sci **49**(1): 81-6.
Helmink, S. K., S. L. Rodriguez-Zas, et al. (2001). "Estimated genetic parameters for growth traits of German shepherd dog and Labrador retriever dog guides." J Anim Sci **79**(6): 1450-6.
Helmink, S. K., R. D. Shanks, et al. (2000). "Breed and sex differences in growth curves for two breeds of dog guides." J Anim Sci **78**(1): 27-32.
Henze, M. J., F. Schaeffel, et al. (2004). "Variations in the off-axis refractive state in the eye of the Vietnamese leaf turtle (Geoemyda spengleri)." J Comp Physiol A Neuroethol Sens Neural Behav Physiol **190**(2): 131-7.
Herraez, D. L. and M. Stoneking (2008). "High fractions of exogenous DNA in human buccal samples reduce the quality of large-scale genotyping." Anal Biochem **383**(2): 329-31.
Hess, C. H., L. (1898). "Arbeiten aus dem Gebiet der Akkommodationslehre
" Arch Ophthalmol **46**: 243-276.
Hine, M. L. (1949). May and Worth's Manual of Diseases of the Eye, Bailliere, Tindall & Cox.
Holle, D. M., M. E. Stankovics, et al. (1999). "The geographic form of retinal dysplasia in dogs is not always a congenital abnormality." Vet Ophthalmol **2**(1): 61-66.
Holmstrom, M., M. el Azazi, et al. (1998). "Ophthalmological long-term follow up of preterm infants: a population based, prospective study of the refraction and its development." Br J Ophthalmol **82**(11): 1265-71.
Howland, H. C. and N. Sayles (1985). "Photokeratometric and photorefractive measurements of astigmatism in infants and young children." Vision Res **25**(1): 73-81.
Howlett, M. H. and S. A. McFadden (2006). "Form-deprivation myopia in the guinea pig (Cavia porcellus)." Vision Res **46**(1-2): 267-83.
Huang, J. and V. J. Vieland (2001). "Comparison of 'model-free' and 'model-based' linkage statistics in the presence of locus heterogeneity: single data set and multiple data set applications." Hum Hered **51**(4): 217-25.
Hung, L. F., M. L. Crawford, et al. (1995). "Spectacle lenses alter eye growth and the refractive status of young monkeys." Nat Med **1**(8): 761-5.

Hunter, L. S., D. J. Sidjanin, et al. (2006). "Radiation hybrid mapping of cataract genes in the dog." Mol Vis **12**: 588-96.
Hur, Y. M., Y. Zheng, et al. (2009). "Comparisons of refractive errors between twins and singletons in Chinese school-age samples." Twin Res Hum Genet **12**(1): 86-92.
Huynh, S. C., A. Kifley, et al. (2006). "Astigmatism and its components in 6-year-old children." Invest Ophthalmol Vis Sci **47**(1): 55-64.
Huynh, S. C., X. Y. Wang, et al. (2006). "Prevalence and associations of anisometropia and aniso-astigmatism in a population based sample of 6 year old children." Br J Ophthalmol **90**(5): 597-601.
Hyman, L. (2007). "Myopic and hyperopic refractive error in adults: an overview." Ophthalmic Epidemiol **14**(4): 192-7.
Inamori, Y., M. Ota, et al. (2007). "The COL1A1 gene and high myopia susceptibility in Japanese." Hum Genet **122**(2): 151-7.
Ip, J. M., S. C. Huynh, et al. (2007). "Ethnic differences in the impact of parental myopia: findings from a population-based study of 12-year-old Australian children." Invest Ophthalmol Vis Sci **48**(6): 2520-8.
Iribarren, R., A. Balsa, et al. (2005). "Family history of myopia is not related to the final amount of refractive error in low and moderate myopia." Clin Experiment Ophthalmol **33**(3): 274-8.
Irving, E. L., J. G. Sivak, et al. (1992). "Refractive plasticity of the developing chick eye." Ophthalmic Physiol Opt **12**(4): 448-56.
Iuvone, P. M., M. Tigges, et al. (1991). "Effects of apomorphine, a dopamine receptor agonist, on ocular refraction and axial elongation in a primate model of myopia." Invest Ophthalmol Vis Sci **32**(5): 1674-7.
Jackson, H. A., T. Olivry, et al. (2004). "Immunopathology of vesicular cutaneous lupus erythematosus in the rough collie and Shetland sheepdog: a canine homologue of subacute cutaneous lupus erythematosus in humans." Vet Dermatol **15**(4): 230-9.
Jacobs, G. H., J. F. Deegan, 2nd, et al. (1993). "Photopigments of dogs and foxes and their implications for canid vision." Vis Neurosci **10**(1): 173-80.
Jacobsen, N., H. Jensen, et al. (2008). "Does the level of physical activity in university students influence development and progression of myopia?--a 2-year prospective cohort study." Invest Ophthalmol Vis Sci **49**(4): 1322-7.
Janutta, V., H. Hamann, et al. (2006). "Complex segregation analysis of canine hip dysplasia in German shepherd dogs." J Hered **97**(1): 13-20.
Johnson, G. L. S., E. (1901). "Contributions to the comparative anatomy of the mammalian eye, based chiefly on ophthalmoscopic examination. Phil. Trans. Royal Soceity. ." **B194**: 1-82.
Jones, L. A., L. T. Sinnott, et al. (2007). "Parental history of myopia, sports and outdoor activities, and future myopia." Invest Ophthalmol Vis Sci **48**(8): 3524-32.
Junghans, B. M. and S. G. Crewther (2005). "Little evidence for an epidemic of myopia in Australian primary school children over the last 30 years." BMC Ophthalmol **5**(1): 1.
Karlsson, E. K., I. Baranowska, et al. (2007). "Efficient mapping of mendelian traits in dogs through genome-wide association." Nat Genet **39**(11): 1321-8.
Karlsson, J. I. (1974). "Concordance rates for myopia in twins." Clin Genet **6**(2): 142-6.
Katz, J., J. M. Tielsch, et al. (1997). "Prevalence and risk factors for refractive errors in an adult inner city population." Invest Ophthalmol Vis Sci **38**(2): 334-40.
Keller, C. A. and R. P. Nugent (1983). "Seasonal patterns in perinatal mortality and preterm delivery." Am J Epidemiol **118**(5): 689-98.
Kirkpatrick, B., E. Messias, et al. (2008). "Schizoid-like features and season of birth in a nonpatient sample." Schizophr Res **103**(1-3): 151-5.

Klein, A. P., P. Duggal, et al. (2007). "Confirmation of linkage to ocular refraction on chromosome 22q and identification of a novel linkage region on 1q." Arch Ophthalmol **125**(1): 80-5.
Klein, A. P., B. Suktitipat, et al. (2009). "Heritability analysis of spherical equivalent, axial length, corneal curvature, and anterior chamber depth in the Beaver Dam Eye Study." Arch Ophthalmol **127**(5): 649-55.
Knobloch, W. H. (1975). "Inherited hyaloideoretinopathy and skeletal dysplasia." Trans Am Ophthalmol Soc **73**: 417-51.
Knobloch, W. H., N. M. Leavenworth, et al. (1985). "Eye findings in twins reared apart." Ophthalmic Paediatr Genet **5**(1-2): 59-66.
Koch, S. A. and L. F. Rubin (1972). "Distribution of cones in retina of the normal dog." Am J Vet Res **33**(2): 361-3.
Konstantopoulos, A., G. Yadegarfar, et al. (2008). "Near work, education, family history, and myopia in Greek conscripts." Eye **22**(4): 542-6.
Kraijer-Huver, I. M., E. J. Gubbels, et al. (2008). "Characterization and prevalence of cataracts in Labrador Retrievers in The Netherlands." Am J Vet Res **69**(10): 1336-40.
Krause, U. H., P. T. Rantakallio, et al. (1993). "The development of myopia up to the age of twenty and a comparison of refraction in parents and children." Arctic Med Res **52**(4): 161-5.
Kruglyak, L. and D. A. Nickerson (2001). "Variation is the spice of life." Nat Genet **27**(3): 234-6.
Kubai, M. A., E. Bentley, et al. (2008). "Refractive states of eyes and association between ametropia and breed in dogs." Am J Vet Res **69**(7): 946-51.
Kukekova, A. V., J. Nelson, et al. (2006). "Linkage mapping of canine rod cone dysplasia type 2 (rcd2) to CFA7, the canine orthologue of human 1q32." Invest Ophthalmol Vis Sci **47**(3): 1210-5.
Kuo, A., R. B. Sinatra, et al. (2003). "Distribution of refractive error in healthy infants." J AAPOS **7**(3): 174-7.
Kurtz, D., L. Hyman, et al. (2007). "Role of parental myopia in the progression of myopia and its interaction with treatment in COMET children." Invest Ophthalmol Vis Sci **48**(2): 562-70.
LaFramboise, T. (2009). "Single nucleotide polymorphism arrays: a decade of biological, computational and technological advances." Nucleic Acids Res **37**(13): 4181-93.
Lam, C. Y., P. O. Tam, et al. (2008). "A genome-wide scan maps a novel high myopia locus to 5p15." Invest Ophthalmol Vis Sci **49**(9): 3768-78.
Lam, D. S., D. S. Fan, et al. (2008). "The effect of parental history of myopia on children's eye size and growth: results of a longitudinal study." Invest Ophthalmol Vis Sci **49**(3): 873-6.
Langston, A. A., C. S. Mellersh, et al. (1997). "Construction of a panel of canine-rodent hybrid cell lines for use in partitioning of the canine genome." Genomics **46**(3): 317-25.
Langston, A. A., C. S. Mellersh, et al. (1999). "Toward a framework linkage map of the canine genome." J Hered **90**(1): 7-14.
Lazar, J., C. Moreno, et al. (2005). "Impact of genomics on research in the rat." Genome Res **15**(12): 1717-28.
Lee, K. E., B. E. Klein, et al. (2001). "Aggregation of refractive error and 5-year changes in refractive error among families in the Beaver Dam Eye Study." Arch Ophthalmol **119**(11): 1679-85.
Lee, K. E., B. E. Klein, et al. (2009). "Association of age, stature, and education with ocular dimensions in an older white population." Arch Ophthalmol **127**(1): 88-93.

Lee, K. E., B. E. Klein, et al. (2002). "Changes in refraction over 10 years in an adult population: the Beaver Dam Eye study." Invest Ophthalmol Vis Sci **43**(8): 2566-71.
Lee, S. J., P. J. Steer, et al. (2006). "Seasonal patterns and preterm birth: a systematic review of the literature and an analysis in a London-based cohort." BJOG **113**(11): 1280-8.
Legendre, P. L., L (1998). Numerical Ecology, Elsevier.
Lesiuk, T. P. and C. R. Braekevelt (1983). "Fine structure of the canine tapetum lucidum." J Anat **136**(Pt 1): 157-64.
Leutenegger, A. L., E. Genin, et al. (2002). "Impact of parental relationships in maximum lod score affected sib-pair method." Genet Epidemiol **23**(4): 413-25.
Li, T., D. Troilo, et al. (1995). "Constant light produces severe corneal flattening and hyperopia in chickens." Vision Res **35**(9): 1203-9.
Li, Y. J., J. A. Guggenheim, et al. (2009). "An international collaborative family-based whole-genome linkage scan for high-grade myopia." Invest Ophthalmol Vis Sci **50**(7): 3116-27.
Li, Z., H. Cui, et al. (2008). "Prevalence and causes of blindness and visual impairment among the elderly in rural southern Harbin, China." Ophthalmic Epidemiol **15**(5): 334-8.
Liang, C. L., E. Yen, et al. (2004). "Impact of family history of high myopia on level and onset of myopia." Invest Ophthalmol Vis Sci **45**(10): 3446-52.
Liang, Y. B., D. S. Friedman, et al. (2008). "Prevalence and causes of low vision and blindness in a rural chinese adult population: the Handan Eye Study." Ophthalmology **115**(11): 1965-72.
Lim, M. C., G. Gazzard, et al. (2009). "Direct costs of myopia in Singapore." Eye **23**(5): 1086-9.
Lim, R., P. Mitchell, et al. (1999). "Refractive associations with cataract: the Blue Mountains Eye Study." Invest Ophthalmol Vis Sci **40**(12): 3021-6.
Lin, L. L. and C. J. Chen (1987). "Twin study on myopia." Acta Genet Med Gemellol (Roma) **36**(4): 535-40.
Lin, L. L., Y. F. Shih, et al. (2004). "Prevalence of myopia in Taiwanese schoolchildren: 1983 to 2000." Ann Acad Med Singapore **33**(1): 27-33.
Lindblad-Toh, K., C. M. Wade, et al. (2005). "Genome sequence, comparative analysis and haplotype structure of the domestic dog." Nature **438**(7069): 803-19.
Lopes, M. C., T. Andrew, et al. (2009). "Estimating heritability and shared environmental effects for refractive error in twin and family studies." Invest Ophthalmol Vis Sci **50**(1): 126-31.
Lowe, J. K., A. V. Kukekova, et al. (2003). "Linkage mapping of the primary disease locus for collie eye anomaly." Genomics **82**(1): 86-95.
Lu, B., N. Congdon, et al. (2009). "Associations between near work, outdoor activity, and myopia among adolescent students in rural China: the Xichang Pediatric Refractive Error Study report no. 2." Arch Ophthalmol **127**(6): 769-75.
Luft, W. A., P. M. Iuvone, et al. (2004). "Spatial, temporal, and intensive determinants of dopamine release in the chick retina." Vis Neurosci **21**(4): 627-35.
Lyhne, N., A. K. Sjolie, et al. (2001). "The importance of genes and environment for ocular refraction and its determiners: a population based study among 20-45 year old twins." Br J Ophthalmol **85**(12): 1470-6.
Macfarlane, D. F., WJ. Stark, DJ. (1987). "The prevalence of ocular disorders in 1000 Queensland primary school children." ANZ Journal Ophthalmol **15**: 161-174.
Maffei, L. F., A. (1981). "Electroretinographic responses to alternating gratings before and after section of the optic nerve. ." Science **211**: 933-935.

Makinen, V. P., M. Parkkonen, et al. (2005). "High-throughput pedigree drawing." Eur J Hum Genet **13**(8): 987-9.
Mandel, Y., I. Grotto, et al. (2008). "Season of birth, natural light, and myopia." Ophthalmology **115**(4): 686-92.
Marg, E. (1954). "Accommodative response of the eye to electrical stimulation of the ciliary ganglion in cats." Am. J. Optom. **31**(3): 127-137.
Mash, A. J., J. P. Hegmann, et al. (1975). "Genetic analysis of indices of corneal power and corneal astigmatism in human populations with varying incidences of strabismus." Invest Ophthalmol **14**(11): 826-32.
Matsuda, S. and H. Kahyo (1992). "Seasonality of preterm births in Japan." Int J Epidemiol **21**(1): 91-100.
Mayer, D. L., R. M. Hansen, et al. (2001). "Cycloplegic refractions in healthy children aged 1 through 48 months." Arch Ophthalmol **119**(11): 1625-8.
McBrien, N. A. and D. A. Barnes (1984). "A review and evaluation of theories of refractive error development." Ophthalmic Physiol Opt **4**(3): 201-13.
McBrien, N. A., A. Gentle, et al. (1999). "Optical correction of induced axial myopia in the tree shrew: implications for emmetropization." Optom Vis Sci **76**(6): 419-27.
McBrien, N. A., H. O. Moghaddam, et al. (1993). "Experimental myopia in a diurnal mammal (Sciurus carolinensis) with no accommodative ability." J Physiol **469**: 427-41.
McCarty, C. A., B. N. Mukesh, et al. (1999). "The epidemiology of cataract in Australia." Am J Ophthalmol **128**(4): 446-65.
McGrath, J. J., A. G. Barnett, et al. (2005). "The association between birth weight, season of birth and latitude." Ann Hum Biol **32**(5): 547-59.
McGreevy, P., T. D. Grassi, et al. (2004). "A strong correlation exists between the distribution of retinal ganglion cells and nose length in the dog." Brain Behav Evol **63**(1): 13-22.
McMahon, G., T. Zayats, et al. (2009). "Season of birth, daylight hours at birth, and high myopia." Ophthalmology **116**(3): 468-73.
Megaw, P. L., I. G. Morgan, et al. (1997). "Dopaminergic behaviour in chicken retina and the effect of form deprivation." Aust N Z J Ophthalmol **25 Suppl 1**: S76-8.
Mellersh, C. S., C. Hitte, et al. (2000). "An integrated linkage-radiation hybrid map of the canine genome." Mamm Genome **11**(2): 120-30.
Mellersh, C. S., A. A. Langston, et al. (1997). "A linkage map of the canine genome." Genomics **46**(3): 326-36.
Metlapally, R., Y. J. Li, et al. (2008). "Common MFRP sequence variants are not associated with moderate to high hyperopia, isolated microphthalmia, and high myopia." Mol Vis **14**: 387-93.
Metlapally, S. and N. A. McBrien (2008). "The effect of positive lens defocus on ocular growth and emmetropization in the tree shrew." J Vis **8**(3): 1 1-12.
Meyer, C., M. F. Mueller, et al. (1999). "Experimental animal myopia models are applicable to human juvenile-onset myopia." Surv Ophthalmol **44 Suppl 1**: S93-102.
Meyers, V. N., P. F. Jezyk, et al. (1983). "Short-limbed dwarfism and ocular defects in the Samoyed dog." J Am Vet Med Assoc **183**(9): 975-9.
Miller, J. M. (2009). "Clinical applications of power vectors." Optom Vis Sci **86**(6): 599-602.
Miller, P. E. and C. J. Murphy (1995). "Vision in dogs." J Am Vet Med Assoc **207**(12): 1623-34.
Montes-Mico, R. and T. Ferrer-Blasco (2000). "Distribution of refractive errors in Spain." Doc Ophthalmol **101**(1): 25-33.

Morgan, M. W. O., J.M. Wartrous, W.G. (1939). "Sympathetic action in accommodation for far vision." Am J Ophthalmol: 588-591.
Morton, N. E. (1955). "Sequential tests for the detection of linkage." American Journal of Human Genetics **7**: 277-318.
Mowat, F. M., S. M. Petersen-Jones, et al. (2008). "Topographical characterization of cone photoreceptors and the area centralis of the canine retina." Mol Vis **14**: 2518-27.
Mrugacz, M., M. Rydzanicz, et al. (2009). "[Myopia in systemic disorders]." Klin Oczna **111**(1-3): 84-8.
Murphy, C. J., R. W. Bellhorn, et al. (1990). "Refractive state, ocular anatomy, and accommodative range of the sea otter (Enhydra lutris)." Vision Res **30**(1): 23-32.
Murphy, C. J., D. O. Mutti, et al. (1997). "Effect of optical defocus on visual acuity in dogs." Am J Vet Res **58**(4): 414-8.
Murphy, C. J., K. Zadnik, et al. (1992). "Myopia and refractive error in dogs." Invest Ophthalmol Vis Sci **33**(8): 2459-63.
Murphy, W. J., B. Davis, et al. (2007). "A 1.5-Mb-resolution radiation hybrid map of the cat genome and comparative analysis with the canine and human genomes." Genomics **89**(2): 189-96.
Murray, L. J., D. P. O'Reilly, et al. (2000). "Season and outdoor ambient temperature: effects on birth weight." Obstet Gynecol **96**(5 Pt 1): 689-95.
Mutembei, H. M., E. R. Mutiga, et al. (2000). "A retrospective study on some reproductive parameters of German shepherd bitches in Kenya." J S Afr Vet Assoc **71**(2): 115-7.
Mutembei, H. M., E. R. Mutiga, et al. (2002). "An epidemiological survey demonstrating decline in reproductive efficiency with age and non-seasonality of reproductive parameters in German shepherd bitches in Kenya." J S Afr Vet Assoc **73**(1): 36-7.
Mutti, D. O., M. E. Cooper, et al. (2007). "Candidate gene and locus analysis of myopia." Mol Vis **13**: 1012-9.
Mutti, D. O., G. L. Mitchell, et al. (2005). "Axial growth and changes in lenticular and corneal power during emmetropization in infants." Invest Ophthalmol Vis Sci **46**(9): 3074-80.
Mutti, D. O., G. L. Mitchell, et al. (2002). "Parental myopia, near work, school achievement, and children's refractive error." Invest Ophthalmol Vis Sci **43**(12): 3633-40.
Mutti, D. O., J. N. Ver Hoeve, et al. (1997). "The artifact of retinoscopy revisited: comparison of refractive error measured by retinoscopy and visual evoked potential in the rat." Optom Vis Sci **74**(7): 483-8.
Mutti, D. O. and K. Zadnik (1995). "The utility of three predictors of childhood myopia: a Bayesian analysis." Vision Res **35**(9): 1345-52.
Mutti, D. O., K. Zadnik, et al. (1999). "Naturally occurring vitreous chamber-based myopia in the Labrador retriever." Invest Ophthalmol Vis Sci **40**(7): 1577-84.
Nagayasu, A., T. Hirayanagi, et al. (2009). "Site-dependent differences in collagen lamellae in the corneal substantia propria of beagle dogs." J Vet Med Sci **71**(9): 1229-31.
Naiglin, L., C. Gazagne, et al. (2002). "A genome wide scan for familial high myopia suggests a novel locus on chromosome 7q36." J Med Genet **39**(2): 118-24.
Nakanishi, H., R. Yamada, et al. (2009). "Absence of association between COL1A1 polymorphisms and high myopia in the Japanese population." Invest Ophthalmol Vis Sci **50**(2): 544-50.
Nallasamy, S., P. C. Paluru, et al. (2007). "Genetic linkage study of high-grade myopia in a Hutterite population from South Dakota." Mol Vis **13**: 229-36.
Neff, M. W., K. W. Broman, et al. (1999). "A second-generation genetic linkage map of the domestic dog, Canis familiaris." Genetics **151**(2): 803-20.

Neuhaus, W. R., E. (1967). "Uber die Sehschaefe des Haushundes bei verschiedenen." Z. Vergl. Physiol. **57**: 137-146

Neuhausen, S. L. (1999). "Ethnic differences in cancer risk resulting from genetic variation." Cancer **86**(11 Suppl): 2575-82.

Nishizaki, R., M. Ota, et al. (2009). "New susceptibility locus for high myopia is linked to the uromodulin-like 1 (UMODL1) gene region on chromosome 21q22.3." Eye (Lond) **23**(1): 222-9.

Nordborg, M. (2000). "Linkage disequilibrium, gene trees and selfing: an ancestral recombination graph with partial self-fertilization." Genetics **154**(2): 923-9.

Norton, T. T. and N. A. McBrien (1992). "Normal development of refractive state and ocular component dimensions in the tree shrew (Tupaia belangeri)." Vision Res **32**(5): 833-42.

Norton, T. T., W. W. Wu, et al. (2003). "Refractive state of tree shrew eyes measured with cortical visual evoked potentials." Optom Vis Sci **80**(9): 623-31.

Nowak, M. R. and W. Neumann (1987). "[Refraction of the dog eye]." Klin Monatsbl Augenheilkd **191**(1): 81-3.

Nurnberg, G., F. K. Jacobi, et al. (2008). "Refinement of the MYP3 locus on human chromosome 12 in a German family with Mendelian autosomal dominant high-grade myopia by SNP array mapping." Int J Mol Med **21**(4): 429-38.

O'Connor, A. R., C. M. Wilson, et al. (2007). "Ophthalmological problems associated with preterm birth." Eye **21**(10): 1254-60.

Oberbauer, A. M., D. I. Grossman, et al. (2003). "Alternatives to blood as a source of DNA for large-scale scanning studies of canine genome linkages." Vet Res Commun **27**(1): 27-38.

Odom, J. V., N. M. Bromberg, et al. (1983). "Canine visual acuity: retinal and cortical field potentials evoked by pattern stimulation." Am J Physiol **245**(5 Pt 1): R637-41.

Okkens, A. C., J. M. Teunissen, et al. (2001). "Influence of litter size and breed on the duration of gestation in dogs." J Reprod Fertil Suppl **57**: 193-7.

Olavarrieta, L., C. Morales-Angulo, et al. (2008). "Stickler and branchio-oto-renal syndromes in a patient with mutations in EYA1 and COL2A1 genes." Clin Genet **73**(3): 262-7.

Ollier, W. E., L. J. Kennedy, et al. (2001). "Dog MHC alleles containing the human RA shared epitope confer susceptibility to canine rheumatoid arthritis." Immunogenetics **53**(8): 669-73.

OMIM (2009). Online Mendelian Inheritance in Man (OMIM), McKusick-Nathans Institute of Genetic Medicine.

Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information.

National Library of Medicine (Bethesda, MD).

Ortega-Pacheco, A., J. C. Segura-Correa, et al. (2006). "Reproductive patterns of stray male dogs in the tropics." Theriogenology **66**(9): 2084-90.

Ostrander, E. A. and K. E. Comstock (2004). "The domestic dog genome." Curr Biol **14**(3): R98-9.

Ostrander, E. A., F. Galibert, et al. (2000). "Canine genetics comes of age." Trends Genet **16**(3): 117-24.

Ostrander, E. A. and E. Giniger (1999). "Let sleeping dogs lie?" Nat Genet **23**(1): 3-4.

Ostrander, E. A. and L. Kruglyak (2000). "Unleashing the canine genome." Genome Res **10**(9): 1271-4.

Ostrander, E. A., E. Lindblad-Toh, et al. Sequencing the Genome of the Domestic Dog Canis familiaris, Fred Hutchison Cancer Research Center

Whitehead/MIT Center for Genome Research
The Canine Genome Mapping Community.
Ostrander, E. A. G., U. Lindblad-Toh, K. (2006). The Dog and its Genome. Cold Spring Harbour, New York, Cold Spring Harbour
Oyama, M. A. (2008). "Canine heart disease: progress and promise." J Small Anim Pract **49**(11): 549-50.
Paget, S., S. Julia, et al. (2008). "Linkage analysis of high myopia susceptibility locus in 26 families." Mol Vis **14**: 2566-74.
Paget, S., Z. G. Vitezica, et al. (2008). "Heritability of refractive value and ocular biometrics." Exp Eye Res **86**(2): 290-5.
Pak, M. A. (1984). "Ocular refraction and visual contrast sensitivity of the rabbit, determined by the VECP." Vision Res **24**(4): 341-5.
Paluru, P., S. M. Ronan, et al. (2003). "New locus for autosomal dominant high myopia maps to the long arm of chromosome 17." Invest Ophthalmol Vis Sci **44**(5): 1830-6.
Paluru, P. C., S. Nallasamy, et al. (2005). "Identification of a novel locus on 2q for autosomal dominant high-grade myopia." Invest Ophthalmol Vis Sci **46**(7): 2300-7.
Pararajasegaram, R. (1999). "VISION 2020-the right to sight: from strategies to action." Am J Ophthalmol **128**(3): 359-60.
Parker, H. G., L. V. Kim, et al. (2004). "Genetic structure of the purebred domestic dog." Science **304**(5674): 1160-4.
Parker, H. G. and E. A. Ostrander (2005). "Canine Genomics and Genetics: Running with the Pack." PLoS Genet **1**(5): e58.
Patterson, D. F., M. E. Haskins, et al. (1988). "Research on genetic diseases: reciprocal benefits to animals and man." J Am Vet Med Assoc **193**(9): 1131-44.
Peichl, L. (1991). "Catecholaminergic amacrine cells in the dog and wolf retina." Vis Neurosci **7**(6): 575-87.
Peichl, L. (1992). "Topography of ganglion cells in the dog and wolf retina." J Comp Neurol **324**(4): 603-20.
Pendrak, K., T. Nguyen, et al. (1997). "Retinal dopamine in the recovery from experimental myopia." Curr Eye Res **16**(2): 152-7.
Pertile, K. K., M. Schache, et al. (2008). "Assessment of TGIF as a candidate gene for myopia." Invest Ophthalmol Vis Sci **49**(1): 49-54.
Pretterer, G., H. Bubna-Littitz, et al. (2004). "Brightness discrimination in the dog." J Vis **4**(3): 241-9.
Price, T. D. G., A.B. (1995). Last hunters-first farmers. New perspectives on the prehistoric transition to agriculture. . Santa Fe, New Mexico, School of American Research Press.
Quinn, G. E., V. Dobson, et al. (1998). "Prevalence of myopia between 3 months and 5 1/2 years in preterm infants with and without retinopathy of prematurity. Cryotherapy for Retinopathy of Prematurity Cooperative Group." Ophthalmology **105**(7): 1292-300.
Quinn, G. E., C. H. Shin, et al. (1999). "Myopia and ambient lighting at night." Nature **399**(6732): 113-4.
Rahi, J. S. and C. Dezateux (2000). "Congenital and infantile cataract in the United Kingdom: underlying or associated factors. British Congenital Cataract Interest Group." Invest Ophthalmol Vis Sci **41**(8): 2108-14.
Raviola, E. and T. N. Wiesel (1985). "An animal model of myopia." N Engl J Med **312**(25): 1609-15.
Ray, K., V. J. Baldwin, et al. (1995). "Molecular diagnostic tests for ascertainment of genotype at the rod cone dysplasia 1 (rcd1) locus in Irish setters." Curr Eye Res **14**(3): 243-7.

Reif, J. S., C. Bruns, et al. (1998). "Cancer of the nasal cavity and paranasal sinuses and exposure to environmental tobacco smoke in pet dogs." Am J Epidemiol **147**(5): 488-92.
Reimann, N., S. Bartnitzke, et al. (1996). "An extended nomenclature of the canine karyotype." Cytogenet Cell Genet **73**(1-2): 140-4.
Rogers, N. L., S. A. Cole, et al. (2007). "New saliva DNA collection method compared to buccal cell collection techniques for epidemiological studies." Am J Hum Biol **19**(3): 319-26.
Roorda, A., M. C. Campbell, et al. (1997). "Slope-based eccentric photorefraction: theoretical analysis of different light source configurations and effects of ocular aberrations." J Opt Soc Am A Opt Image Sci Vis **14**(10): 2547-56.
Rose, K., W. Smith, et al. (2001). "The increasing prevalence of myopia: implications for Australia." Clin Experiment Ophthalmol **29**(3): 116-20.
Rose, K. A., I. G. Morgan, et al. (2008). "Outdoor Activity Reduces the Prevalence of Myopia in Children." Ophthalmology.
Rose, K. A., I. G. Morgan, et al. (2002). "High heritability of myopia does not preclude rapid changes in prevalence." Clin Experiment Ophthalmol **30**(3): 168-72.
Rosenfield, M. and B. Gilmartin (1987). "Effect of a near-vision task on the response AC/A of a myopic population." Ophthalmic Physiol Opt **7**(3): 225-33.
Rosenfield, M. and B. Gilmartin (1998). Myopia and near work, Butterworth-Heinemann.
Rudnicka, A. R., C. G. Owen, et al. (2008). "Effect of breastfeeding and sociodemographic factors on visual outcome in childhood and adolescence." Am J Clin Nutr **87**(5): 1392-9.
Rylander-Rudqvist, T., N. Hakansson, et al. (2006). "Quality and quantity of saliva DNA obtained from the self-administrated oragene method--a pilot study on the cohort of Swedish men." Cancer Epidemiol Biomarkers Prev **15**(9): 1742-5.
Salomao, S. R., M. R. Mitsuhiro, et al. (2009). "Visual impairment and blindness: an overview of prevalence and causes in Brazil." An Acad Bras Cienc **81**(3): 539-49.
Sargan, D. R. (2004). "IDID: inherited diseases in dogs: web-based information for canine inherited disease genetics." Mamm Genome **15**(6): 503-6.
Sargan, D. R., J. Aguirre-Hernandez, et al. (2007). "An extended microsatellite set for linkage mapping in the domestic dog." J Hered **98**(3): 221-31.
Saunders, K. J. (1995). "Early refractive development in humans." Surv Ophthalmol **40**(3): 207-16.
Saunders, K. J., D. L. McCulloch, et al. (2002). "Emmetropisation following preterm birth." Br J Ophthalmol **86**(9): 1035-40.
Saunders, K. J., J. M. Woodhouse, et al. (1995). "Emmetropisation in human infancy: rate of change is related to initial refractive error." Vision Res **35**(9): 1325-8.
Saw, S. M. (2006). "How blinding is pathological myopia?" Br J Ophthalmol **90**(5): 525-6.
Saw, S. M. and S. J. Chew (1997). "Myopia in children born premature or with low birth weight." Acta Ophthalmol Scand **75**(5): 548-50.
Saw, S. M., G. Gazzard, et al. (2002). "Myopia: attempts to arrest progression." Br J Ophthalmol **86**(11): 1306-11.
Saw, S. M., F. J. Nieto, et al. (2001). "Familial clustering and myopia progression in Singapore school children." Ophthalmic Epidemiol **8**(4): 227-36.
Saw, S. M., L. Tong, et al. (2004). "The relation between birth size and the results of refractive error and biometry measurements in children." Br J Ophthalmol **88**(4): 538-42.
Saw, S. M., H. M. Wu, et al. (2001). "Myopia and night lighting in children in Singapore." Br J Ophthalmol **85**(5): 527-8.

Schaeffel, F., E. Burkhardt, et al. (2004). "Measurement of refractive state and deprivation myopia in two strains of mice." Optom Vis Sci **81**(2): 99-110.
Schaeffel, F., G. Hagel, et al. (1994). "Lower-field myopia and astigmatism in amphibians and chickens." J Opt Soc Am A Opt Image Sci Vis **11**(2): 487-95.
Schmid, K. L. and C. F. Wildsoet (1996). "Effects on the compensatory responses to positive and negative lenses of intermittent lens wear and ciliary nerve section in chicks." Vision Res **36**(7): 1023-36.
Schwahn, H. N., H. Kaymak, et al. (2000). "Effects of atropine on refractive development, dopamine release, and slow retinal potentials in the chick." Vis Neurosci **17**(2): 165-76.
Schwartz, M., M. Haim, et al. (1990). "X-linked myopia: Bornholm eye disease. Linkage to DNA markers on the distal part of Xq." Clin Genet **38**(4): 281-6.
Shaikh, A. W., J. T. Siegwart, Jr., et al. (1999). "Effect of interrupted lens wear on compensation for a minus lens in tree shrews." Optom Vis Sci **76**(5): 308-15.
Sharon, D., G. A. Bruns, et al. (2000). "X-linked retinitis pigmentosa: mutation spectrum of the RPGR and RP2 genes and correlation with visual function." Invest Ophthalmol Vis Sci **41**(9): 2712-21.
Shen, W., M. Vijayan, et al. (2005). "Inducing form-deprivation myopia in fish." Invest Ophthalmol Vis Sci **46**(5): 1797-803.
Sherman, S. M., T. T. Norton, et al. (1977). "Myopia in the lid-sutured tree shrew (Tupaia glis)." Brain Res **124**(1): 154-7.
Short, A. D., L. J. Kennedy, et al. (2005). "Canine DNA subjected to whole genome amplification is suitable for a wide range of molecular applications." J Hered **96**(7): 829-35.
Siatkowski, R. M., S. A. Cotter, et al. (2008). "Two-year multicenter, randomized, double-masked, placebo-controlled, parallel safety and efficacy study of 2% pirenzepine ophthalmic gel in children with myopia." J AAPOS **12**(4): 332-9.
Siegwart, J. T., Jr. and T. T. Norton (1998). "The susceptible period for deprivation-induced myopia in tree shrew." Vision Res **38**(22): 3505-15.
Siegwart, J. T., Jr. and T. T. Norton (1999). "Regulation of the mechanical properties of tree shrew sclera by the visual environment." Vision Res **39**(2): 387-407.
Simpson, C. L., P. Hysi, et al. (2007). "The Roles of PAX6 and SOX2 in Myopia: lessons from the 1958 British Birth Cohort." Invest Ophthalmol Vis Sci **48**(10): 4421-5.
Smith, E. L., 3rd, D. V. Bradley, et al. (2001). "Continuous ambient lighting and eye growth in primates." Invest Ophthalmol Vis Sci **42**(6): 1146-52.
Smith, E. L., 3rd, R. S. Harwerth, et al. (1987). "Observations on the effects of form deprivation on the refractive status of the monkey." Invest Ophthalmol Vis Sci **28**(8): 1236-45.
Smith, E. L., 3rd and L. F. Hung (1999). "The role of optical defocus in regulating refractive development in infant monkeys." Vision Res **39**(8): 1415-35.
Smith, E. L., 3rd and L. F. Hung (2000). "Form-deprivation myopia in monkeys is a graded phenomenon." Vision Res **40**(4): 371-81.
Smith, E. L., 3rd, L. F. Hung, et al. (1994). "Effects of optically induced blur on the refractive status of young monkeys." Vision Res **34**(3): 293-301.
Smith, E. L., 3rd, R. Ramamirtham, et al. (2007). "Effects of foveal ablation on emmetropization and form-deprivation myopia." Invest Ophthalmol Vis Sci **48**(9): 3914-22.
Smith, T. S., K. D. Frick, et al. (2009). "Potential lost productivity resulting from the global burden of uncorrected refractive error." Bull World Health Organ **87**(6): 431-7.

Sorsby, A. and G. R. Fraser (1964). "Statistical Note on the Components of Ocular Refraction in Twins." J Med Genet **55**: 47-9.
Sperduto, R. D., D. Seigel, et al. (1983). "Prevalence of myopia in the United States." Arch Ophthalmol **101**(3): 405-7.
Stambolian, D., E. B. Ciner, et al. (2005). "Genome-wide scan for myopia in the Old Order Amish." Am J Ophthalmol **140**(3): 469-76.
Stambolian, D., G. Ibay, et al. (2004). "Genomewide linkage scan for myopia susceptibility loci among Ashkenazi Jewish families shows evidence of linkage on chromosome 22q12." Am J Hum Genet **75**(3): 448-59.
Stambolian, D., G. Ibay, et al. (2006). "Genome-wide scan of additional Jewish families confirms linkage of a myopia susceptibility locus to chromosome 22q12." Mol Vis **12**: 1499-505.
Starkey, M. P., T. J. Scase, et al. (2005). "Dogs really are man's best friend--canine genomics has applications in veterinary and human medicine!" Brief Funct Genomic Proteomic **4**(2): 112-28.
Stieger, K. and B. Lorenz (2008). "[The treatment of inherited dystrophies and neovascular disorders of the retina by rAAV-mediated gene therapy]." Klin Monbl Augenheilkd **225**(12): 1009-23.
Stoltenberg, S. F. (1997). "Coming to terms with heritability." Genetica **99**(2-3): 89-96.
Stone, R. A., T. Lin, et al. (1995). "Photoperiod, early post-natal eye growth, and visual deprivation." Vision Res **35**(9): 1195-202.
Stone, R. A., T. Lin, et al. (1989). "Retinal dopamine and form-deprivation myopia." Proc Natl Acad Sci U S A **86**(2): 704-6.
Strain, G. M., L. A. Clark, et al. (2009). "Prevalence of deafness in dogs heterozygous or homozygous for the merle allele." J Vet Intern Med **23**(2): 282-6.
Switonski, M., N. Reimann, et al. (1996). "Report on the progress of standardization of the G-banded canine (Canis familiaris) karyotype. Committee for the Standardized Karyotype of the Dog (Canis familiaris)." Chromosome Res **4**(4): 306-9.
Tang, D., R. M. Santella, et al. (1995). "A molecular epidemiological case-control study of lung cancer." Cancer Epidemiol Biomarkers Prev **4**(4): 341-6.
Tarpataki, N., B. Bigler, et al. (2008). "Comparison between an intradermal skin test and allergen-specific IgE-ELISA for canine atopic dermatitis." Schweiz Arch Tierheilkd **150**(3): 117-22.
Tedor, J. B. and J. S. Reif (1978). "Natal patterns among registered dogs in the United States." J Am Vet Med Assoc **172**(10): 1179-85.
Teikari, J., M. Koskenvuo, et al. (1990). "Study of gene-environment effects on development of hyperopia: a study of 191 adult twin pairs from the Finnish Twin Cohort Study." Acta Genet Med Gemellol (Roma) **39**(1): 133-6.
Teikari, J. M. (1987). "Myopia and stature." Acta Ophthalmol (Copenh) **65**(6): 673-6.
Teikari, J. M., J. Kaprio, et al. (1992). "Heritability of defects of far vision in young adults--a twin study." Scand J Soc Med **20**(2): 73-8.
Thibos, L. N., W. Wheeler, et al. (1997). "Power vectors: an application of Fourier analysis to the description and statistical analysis of refractive error." Optom Vis Sci **74**(6): 367-75.
Thomas, R., S. E. Duke, et al. (2008). "A genome assembly-integrated dog 1 Mb BAC microarray: a cytogenetic resource for canine cancer studies and comparative genomic analysis." Cytogenet Genome Res **122**(2): 110-21.
Thompson, E. A. and R. G. Shaw (1990). "Pedigree analysis for quantitative traits: variance components without matrix inversion." Biometrics **46**(2): 399-413.

Thornton, T. and M. S. McPeek (2007). "Case-control association testing with related individuals: a more powerful quasi-likelihood score test." Am J Hum Genet **81**(2): 321-37.
Trangerud, C., J. Grondalen, et al. (2007). "A longitudinal study on growth and growth variables in dogs of four large breeds raised in domestic environments." J Anim Sci **85**(1): 76-83.
Troilo, D. (1992). "Neonatal eye growth and emmetropisation--a literature review." Eye (Lond) **6 (Pt 2)**: 154-60.
Troilo, D., M. D. Gottlieb, et al. (1987). "Visual deprivation causes myopia in chicks with optic nerve section." Curr Eye Res **6**(8): 993-9.
Troilo, D. and S. J. Judge (1993). "Ocular development and visual deprivation myopia in the common marmoset (Callithrix jacchus)." Vision Res **33**(10): 1311-24.
Troilo, D., D. L. Nickla, et al. (2000). "Form deprivation myopia in mature common marmosets (Callithrix jacchus)." Invest Ophthalmol Vis Sci **41**(8): 2043-9.
Troilo, D. and J. Wallman (1991). "The regulation of eye growth and refractive state: an experimental study of emmetropization." Vision Res **31**(7-8): 1237-50.
Twelker, J. D. and D. O. Mutti (2001). "Retinoscopy in infants using a near noncycloplegic technique, cycloplegia with tropicamide 1%, and cycloplegia with cyclopentolate 1%." Optom Vis Sci **78**(4): 215-22.
van der Beek, S., A. L. Nielen, et al. (1999). "Evaluation of genetic, common-litter, and within-litter effects on preweaning mortality in a birth cohort of puppies." Am J Vet Res **60**(9): 1106-10.
Vannas, A. E., G. S. Ying, et al. (2003). "Myopia and natural lighting extremes: risk factors in Finnish army conscripts." Acta Ophthalmol Scand **81**(6): 588-95.
Varghese, R. M., V. Sreenivas, et al. (2009). "Refractive status at birth: its relation to newborn physical parameters at birth and gestational age." PLoS One **4**(2): e4469.
Varughese, S., R. M. Varghese, et al. (2005). "Refractive error at birth and its relation to gestational age." Curr Eye Res **30**(6): 423-8.
Vatavuk, Z., J. Skunca Herman, et al. (2009). "Common variant in myocilin gene is associated with high myopia in isolated population of Korcula Island, Croatia." Croat Med J **50**(1): 17-22.
Venter, J. C., M. D. Adams, et al. (2001). "The sequence of the human genome." Science **291**(5507): 1304-51.
Vila, C., J. E. Maldonado, et al. (1999). "Phylogenetic relationships, evolution, and genetic diversity of the domestic dog." J Hered **90**(1): 71-7.
Vila, C., P. Savolainen, et al. (1997). "Multiple and ancient origins of the domestic dog." Science **276**(5319): 1687-9.
Visscher, P. M., T. Andrew, et al. (2008). "Genome-wide association studies of quantitative traits with related individuals: little (power) lost but much to be gained." Eur J Hum Genet **16**(3): 387-90.
Vitzthum, V. J. (2003). "A number no greater than the sum of its parts: the use and abuse of heritability." Hum Biol **75**(4): 539-58.
Vongphanit, J., P. Mitchell, et al. (2002). "Prevalence and progression of myopic retinopathy in an older population." Ophthalmology **109**(4): 704-11.
Wallman, J. and J. I. Adams (1987). "Developmental aspects of experimental myopia in chicks: susceptibility, recovery and relation to emmetropization." Vision Res **27**(7): 1139-63.
Wallman, J., M. D. Gottlieb, et al. (1987). "Local retinal regions control local eye growth and myopia." Science **237**(4810): 73-7.

Wallman, J., J. Turkel, et al. (1978). "Extreme myopia produced by modest change in early visual experience." Science **201**(4362): 1249-51.
Wallman, J. and J. Winawer (2004). "Homeostasis of eye growth and the question of myopia." Neuron **43**(4): 447-68.
Walls, G. L. (1963). The vertebrate eye and its adaptive radiation. . New York, Hafner Publishing.
Wang, D. G., J. B. Fan, et al. (1998). "Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome." Science **280**(5366): 1077-82.
Wang, P., S. Li, et al. (2009). "High myopia is not associated with the SNPs in the TGIF, lumican, TGFB1, and HGF genes." Invest Ophthalmol Vis Sci **50**(4): 1546-51.
Waters, D. J., G. J. Patronek, et al. (1996). "Comparing the Age at Prostate Cancer Diagnosis in Humans and Dogs." Journal of the National Cancer Institute **88**(22): 1686-1687.
Waterston, R. H., K. Lindblad-Toh, et al. (2002). "Initial sequencing and comparative analysis of the mouse genome." Nature **420**(6915): 520-62.
Wattie, N., C. I. Ardern, et al. (2008). "Season of birth and prevalence of overweight and obesity in Canada." Early Hum Dev **84**(8): 539-47.
Wayne, R. K. (1986). "Limb morphology of domestic and wild canids: the influence of development on morphologic change." J Morphol **187**(3): 301-19.
Wayne, R. K. (1993). "Molecular evolution of the dog family." Trends Genet **9**(6): 218-24.
Wayne, R. K., W. G. Nash, et al. (1987). "Chromosomal evolution of the Canidae. II. Divergence from the primitive carnivore karyotype." Cytogenet Cell Genet **44**(2-3): 134-41.
Wayne, R. K. and E. A. Ostrander (2004). "Out of the dog house: the emergence of the canine genome." Heredity **92**(4): 273-4.
Wen, G. Y., J. A. Sturman, et al. (1985). "A comparative study of the tapetum, retina and skull of the ferret, dog and cat." Lab Anim Sci **35**(3): 200-10.
Wensor, M., C. A. McCarty, et al. (1999). "Prevalence and risk factors of myopia in Victoria, Australia." Arch Ophthalmol **117**(5): 658-63.
Werner, P., M. G. Raducha, et al. (1997). "Physical and linkage mapping of human chromosome 17 loci to dog chromosomes 9 and 5." Genomics **42**(1): 74-82.
WHO (May 2009). Visual Impairment and Blindness. Fact Sheets.
Wiesel, T. N. and E. Raviola (1977). "Myopia and eye enlargement after neonatal lid fusion in monkeys." Nature **266**(5597): 66-8.
Wildsoet, C. and J. Wallman (1995). "Choroidal and scleral mechanisms of compensation for spectacle lenses in chicks." Vision Res **35**(9): 1175-94.
Winick, J. D., M. L. Blundell, et al. (1999). "Homozygosity mapping of the Achromatopsia locus in the Pingelapese." Am J Hum Genet **64**(6): 1679-85.
Witkovsky, P. (2004). "Dopamine and retinal function." Doc Ophthalmol **108**(1): 17-40.
Wojciechowski, R., N. Congdon, et al. (2005). "Heritability of refractive error and familial aggregation of myopia in an elderly American population." Invest Ophthalmol Vis Sci **46**(5): 1588-92.
Wojciechowski, R., C. Moy, et al. (2006). "Genomewide scan in Ashkenazi Jewish families demonstrates evidence of linkage of ocular refraction to a QTL on chromosome 1p36." Hum Genet **119**(4): 389-99.
Wong, T. Y., P. J. Foster, et al. (2000). "Prevalence and risk factors for refractive errors in adult Chinese in Singapore." Invest Ophthalmol Vis Sci **41**(9): 2486-94.
Wood, J. L., K. H. Lakhani, et al. (2004). "An epidemiological approach to prevention and control of three common heritable diseases in canine pedigree breeds in the United Kingdom." Vet J **168**(1): 14-27.

Wood, S. H., X. Ke, et al. (2009). "Genome-wide association analysis of canine atopic dermatitis and identification of disease related SNPs." Immunogenetics.
Wright, H. R., J. E. Keeffe, et al. (2009). "Trachoma, cataracts and uncorrected refractive error are still important contributors to visual morbidity in two remote indigenous communities of the Northern Territory, Australia." Clin Experiment Ophthalmol **37**(6): 550-7.
Wright, S. (1922). "Coefficients of inbreeding and relationship." American Naturalist **56**: 330-338.
Wu, M. M. and M. H. Edwards (1999). "The effect of having myopic parents: an analysis of myopia in three generations." Optom Vis Sci **76**(6): 387-92.
Young, F. A. (1964). "The Distribution of Refractive Errors in Monkeys." Exp Eye Res **3**: 230-8.
Young, T. L., S. M. Ronan, et al. (1998). "A second locus for familial high myopia maps to chromosome 12q." Am J Hum Genet **63**(5): 1419-24.
Young, T. L., S. M. Ronan, et al. (1998). "Evidence that a locus for familial high myopia maps to chromosome 18p." Am J Hum Genet **63**(1): 109-19.
Zadnik, K., L. A. Jones, et al. (2000). "Myopia and ambient night-time lighting. CLEERE Study Group. Collaborative Longitudinal Evaluation of Ethnicity and Refractive Error." Nature **404**(6774): 143-4.
Zadnik, K. and D. O. Mutti (1995). "How applicable are animal myopia models to human juvenile onset myopia?" Vision Res **35**(9): 1283-8.
Zadnik, K., D. O. Mutti, et al. (1993). "Initial cross-sectional results from the Orinda Longitudinal Study of Myopia." Optom Vis Sci **70**(9): 750-8.
Zadnik, K., W. A. Satariano, et al. (1994). "The effect of parental history of myopia on children's eye size." JAMA **271**(17): 1323-7.
Zhang, Q., X. Guo, et al. (2005). "A new locus for autosomal dominant high myopia maps to 4q22-q27 between D4S1578 and D4S1612." Mol Vis **11**: 554-60.
Zhang, Q., X. Guo, et al. (2006). "Novel locus for X linked recessive high myopia maps to Xq23-q25 but outside MYP1." J Med Genet **43**(5): e20.
Zheng, Y., J. Ge, et al. (2008). "Heritability of central corneal thickness in Chinese: the Guangzhou Twin Eye Study." Invest Ophthalmol Vis Sci **49**(10): 4303-7.
Zhou, X., J. Qu, et al. (2006). "Normal development of refractive state and ocular dimensions in guinea pigs." Vision Res **46**(18): 2815-23.
Zhu, L., Z. Zhang, et al. (2009). "The long (and winding) road to gene discovery for canine hip dysplasia." Vet J **181**(2): 97-110.
Zhu, X., T. W. Park, et al. (2005). "In a matter of minutes, the eye can know which way to grow." Invest Ophthalmol Vis Sci **46**(7): 2238-41.
Zimin, A. V., A. L. Delcher, et al. (2009). "A whole-genome assembly of the domestic cow, Bos taurus." Genome Biol **10**(4): R42.