INVESTIGATIONS OF A NOVEL RETINAL DISEASE IN WILTSHIRE SHEEP

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ABSTRACT

In 2011 and 2012, nine cases of adult-onset blindness were identified in a single flock of Wiltshire sheep. Affected sheep typically developed detectable night blindness at 2 to 3 years of age, which progressed to complete blindness by 4 to 5 years of age. Opthalmoscopically, the disease was characterised by progressive tapetal hyperreflectivity and attenuation of retinal blood vessels, indicative of retinal thinning and atrophy. Retinal histology revealed a selective loss of rod photoreceptors in the early stages of the disease, with preservation of cone photoreceptors. Secondary loss of cone photoreceptors was seen later in the course of the disease. Retinal degeneration was not accompanied by any other ocular or central nervous system abnormalities.

Progressive retinal degeneration targeting rod photoreceptors has not been previously reported in sheep, but this disease has several similarities to inherited retinal dystrophies in other species, particularly progressive retinal atrophy in dogs and retinitis pigmentosa in humans. The disease in sheep is thought to be inherited in either an autosomal dominant or autosomal recessive manner, although additional cases identified recently provide further support for an autosomal dominant mode of inheritance. Initial investigations into the molecular basis of the disease, using a comparative candidate gene approach, did not identify any exonic single nucleotide polymorphisms (SNPs) in the rhodopsin gene of affected sheep that would alter the amino acid sequence. Homozygosity mapping of affected sheep revealed an identical-by-descent region on chromosome 5, but none of the genes within or surrounding this segment were considered to be plausible candidate genes except for *GPR98,* which is associated with retinitis pigmentosa and sensorineural hearing loss in humans. Investigations into the

inheritance and molecular basis of this novel retinal degeneration in Wiltshire sheep are continuing, as this disease may prove to be a useful model for retinal dystrophies in other species, including retinitis pigmentosa in humans.

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ABBREVIATIONS

adRP	autosomal dominant retinitis pigmentosa
Ardo	Ardo 00-394 ram
arRP	autosomal recessive retinitis pigmentosa
AV	AV 624-06 ram
cGMP	cyclic guanosine monophosphate
CNG channel	cyclic nucleotide-gated channel
CLN	ceroid lipofuscinosis, neuronal
CRB1	crumbs homolog 1 (*Drosophila*) gene
DNA	deoxyribonucleic acid
GNAT1	transducin
GPR98	G-protein coupled receptor 98 gene (also known as VLGR1)
GDP	guanosine diphosphate
G_t	transducin
GTP	guanosine triphosphate
H&E	hematoxylin and eosin stain
HH	HH 03-88 ram
kDa	kilodalton
mRNA	messenger ribonucleic acid
Na	sodium
NCL	neuronal ceroid lipofuscinoses
OAR_v3.1	sheep genome assembly, version 3.1
OAR5	sheep chromosome 5
OAR19	sheep chromosome 19

PRA	progressive retinal atrophy
PAX6	paired box 6 gene
PCR	polymerase chain reaction
PDE	phosophodiesterase
PDE6A	phosphodiesterase 6A, cGMP-specific, rod, alpha gene
PDE6B	phosphodiesterase 6B, cGMP-specific, rod, beta gene
PITX3	paired-like homeodomain 3 gene
PrP^{Sc}	abnormal Scrapie prion protein
RDS	retinal degeneration, slow (peripherin 2) gene
RHO	rhodopsin gene
RP	retinitis pigmentosa
RPE	retinal pigment epithelium
RPE65	retinal pigment epithelium-specific protein 65kDa gene
RPGR	retinitis pigmentosa GTPase regulator gene
Rx	retinal homeobox gene
SARD	sudden acquired retinal degeneration
SB 01	SB 01-W7 ram
SB 02	SB 02-08 ram
SIX3	SIX homeobox 3 gene
SIX6	SIX homeobox 6 gene
SNP	single nucleotide polymorphism
USH2A	Usher syndrome 2A

GLOSSARY

apoptosis	programmed cell death
bright blindness	progressive retinal degeneration due to chronic ingestion of bracken fern containing ptaquiloside
ceroid lipofuscinoses	a group of inherited, neurodegenerative, lysosomal storage diseases
cone photoreceptors	photoreceptors which are primarily function in bright light and are important in colour vision and visual acuity
dominant trait	a genetic trait in which one copy of the gene is sufficient for an individual to display that trait
decussation	crossing of nerve fibres
electroretinography	measurement of electrical responses of different types of cell within the retina, using a system of electrodes
fundic examination	examination of the retina and posterior segment of the eye using an ophthalmoscope
ganglion cells	large multipolar neurons within the retina
heterozygous	different alleles at one locus
homozygous	the same alleles at one locus
homozygosity mapping	a method of mapping recessive traits, using a SNP array to identify homozygous regions that are identical by descent
hyperreflective	increased reflectivity
inner nuclear layer	a layer of the retina containing the cell bodies and nuclei of horizontal cells, bipolar cells, amacrine cells and Müller cells

inner plexiform layer	a layer of synaptic interactions in the retina, consisting of the cell processes of retinal ganglion cells, bipolar neurons and amacrine cells
nyctalopia	night blindness
outer nuclear layer	a layer of the retina which contains the nuclei of photoreceptor cells
outer plexiform layer	a layer of neuronal synapses within the retina
papilloedema	swelling of the optic disc
photopic vision	vision in well-lit conditions
progressive retinal atrophy	the general term for inherited progressive retinal degenerations in dogs
recessive trait	a genetic trait which is expressed in the homozygous state, but not in the presence of a dominant allele
retinitis pigmentosa	a group of hereditary retinal diseases in humans characterised by primary degeneration of rod photoreceptors
retinal dysplasia	developmental abnormality of the retina
retinal dystrophy	a hereditary, progressive retinal disease
retinopathy	acquired retinal disease
rhodopsin	the primary visual pigment associated with rod photoreceptor cells
rod photoreceptors	photoreceptor cells which are primarily responsible for vision in dim light, and the predominant type of photoreceptor in the sheep retina
scotopic vision	vision in low light conditions
single nucleotide polymorphism	a single nucleotide variation in a DNA sequence

status spongiosis	cavitation of the neuropil within a glial meshwork, appearing as irregular fluid filled spaces microscopically
stereopsis	the visual perception of depth, three dimensional vision
tapetum	a layer of tissue within the choroid at the back of the eye, which functions to reflect light not absorbed by photoreceptors
Usher syndrome	a syndrome of sensorineural hearing loss and vision loss due to retinitis pigmentosa (a syndromic form of retinitis pigmentosa)
visual acuity	clearness of vision

Chapter 1

REVIEW OF THE LITERATURE

1.1 INTRODUCTION

Multiple cases of adult-onset blindness were identified in a flock of Wiltshire sheep in Darfield during 2011 and 2012, and preliminary findings were supportive of retinal degeneration. The retina is a complex tissue composed of 10 distinct layers of cells, and an appreciation of retinal development and structure is useful in understanding the pathogenesis of retinal diseases. Accordingly, the first part of this chapter reviews the normal vertebrate retina (section 1.3 and section 1.4). In sheep, there are a number of diseases which can affect the retina, some of which also cause lesions in other organs, and these are reviewed in section 1.5. Subsequent investigations in affected Wiltshire sheep suggested that this disease primarily targets rod photoreceptors and could be inherited. Such a disease has not been previously reported in sheep, but inherited retinopathies are well recognized in other species and these are reviewed in section 1.6. In particular, the

disease in Wiltshire sheep shows numerous similarities with hereditary retinal degenerations in humans, termed retinitis pigmentosa, and progressive retinal atrophy in dogs. The features of retinitis pigmentosa and progressive retinal atrophy are reviewed in section 1.6, and discussed in greater detail in Chapter 2.

1.2 VISION IN SHEEP

The eyes of sheep have a horizontally elliptical pupil, providing a wide visual field that has been estimated to be 314° (Piggins & Phillips, 1996). There is 40 to 60° overlap between monocular fields, enabling binocular vision and possibly coarse stereopsis. Sheep eyes are similar in size, shape, optical power and structure to human eyes, but the interocular distance in sheep is approximately twice that of humans. Static visual acuity is worse in sheep than humans, and it appears as though sheep eyes lack an ability for accommodation (Kendrick, 1990), whereby changes in the lens occur to maintain a clearly focussed image on the retina as an object moves closer or further from the eye.

Blindness in sheep is an undesirable characteristic as it can create management difficulties. However, research using blinkered sheep suggests that blindness has little effect on total food intake and productivity. Sheep that could not see what they were eating maintained the same pasture species preferences, although on short herbage they tended to eat more of the taller components than the sheep that could see (Arnold, 1966). The blind Wiltshire sheep described within this report also seemed to be able to raise lambs effectively, and it has been shown that while sheep use vision for recognition of offspring at a distance, olfactory recognition is relied upon at close range (Piggins & Phillips, 1996).

1.3 DEVELOPMENT OF THE RETINA

The retina is a thin, highly organised layer of neural tissue which lines the posterior aspect of the eye (Dowling, 2002). Vertebrate eyes are designed to produce a well-focused image on the retina, which is transduced into electrical energy by photoreceptors and transmitted to the visual cortex within the brain (Piggins & Phillips, 1996). Embryologically, the retina is derived from neuroectoderm and is a derivative of the forebrain (Boileau & Gilmour, 2012) . During the neural plate stage of embryogenesis, a single optic field area forms, from which bilateral eye fields then develop. Early ocular morphogenesis in vertebrates is controlled by a number of genes including *PAX6, SIX3, SIX6*, and *Rx* (Chow & Lang, 2001). The signalling protein Sonic hedgehog (Shh), secreted by the anterior axial mesoderm, is vital in the formation of bilateral eye fields (Zhang & Yang, 2001b), and this molecule also plays an important role in the regulation of retinal ganglion cell differentiation later in ocular development (Zhang & Yang, 2001a). Inhibition of Shh through ingestion of *Veratum* sp. containing cyclopamine alkaloids by ewes on day 14 of gestation can result in cyclopia, with the development of a single, central eye (de Lahunta & Glass, 2009). Subsequent closure of the anterior end of the neural tube results in the formation of two optic vesicles during the period of organogenesis, which enlarge and bulge to come into contact with the surface ectoderm. The ectoderm thickens at the point of contact with each optic vesicle, to form the lens placode. Co-ordinated invagination of the lens placode and optic vesicle leads to the formation of the lens vesicle and the double-layered optic cup (de Lahunta & Glass, 2009). Abnormal formation of the lens vesicle due to a missense mutation in the homeodomain of the *PITX3* gene can result in microphthalmia, an inherited disease in Texel sheep (Becker *et al.*, 2010). When

development proceeds normally, the lens vesicle separates from surrounding ectoderm to form the lens, while the corneal epithelium develops from the ectoderm (Stone, 1988).

The optic cup is lined by two layers of epithelium and is connected to the prosencephalon by the optic stalk. From the anterior aspect of each optic cup, the anterior uvea develops, while the posterior part of the cup forms the retina. The outer layer of the posterior optic cup is pigmented and forms the retinal pigment epithelium, while the inner layer is non-pigmented and forms the sensory retina (Ofri, 2008a). Neuroepithelial cells of the inner layer proliferate to form the common neuroblastic layer, which divides into the inner and outer neuroblastic cell layers, separated by the transient layer of Chievitz. In the bovine embryo, this division into inner and outer layers occurs at 40-50 days of gestation (Bistner, Rubin, & Aguirre, 1973). The cells of the outer neuroblastic layer differentiate into photoreceptors externally, and horizontal cells internally; while the cells of the inner neuroblastic layer differentiate into ganglion cells, amacrine cells, bipolar cells and Müller's cells (Ofri, 2008a). Studies over the last 30 years have demonstrated that all the aforementioned types of retinal cell are produced from multi-potent retinal progenitor cells, but the multi-potentiality of these progenitors declines as development proceeds (Turner & Cepko, 1986; Wetts & Fraser, 1988). A number of transcription factors, vital for differentiation into particular cell types, are expressed by retinal progenitor and post-mitotic retinal precursor cells at certain stages of retinal development. Most of these transcription factors are of the basic helix-loop-helix and homeodomain families; inactivation, or altered expression of the genes encoding these factors, can lead to marked changes in the cellular composition of the retina (Reese, 2011).

Across species, there is a conserved temporal pattern for the periods of differentiation of each type of retinal cell, although the neurogenetic windows for different cell types do overlap (Reese, 2011). Retinal development can be divided into two phases. The first phase includes genesis of ganglion cells, horizontal cells, cones and amacrine cells, and in this phase cells in the central retina are generated before those in the peripheral retina. As the first phase concludes at the periphery of the retina, the second phase commences in the mid-temporal retina, and it is during this phase that bipolar cells, Müller cells and rods are formed (Harman, Sanderson, & Beazley, 2004). Cell division continues in the periphery of the retina later than it does in the mid-temporal region, resulting in significant lateral expansion in the second phase. Based on assumptions derived from the homochrony hypothesis (Quinlivan *et al.*, 2000; Harman, *et al.*, 2004; la Vail, Rapaport, & Rakic, 2004), the second phase of retinal development is calculated to occur between 71-115 days of gestation in sheep, and the retina thickens during this time. Repeated corticosteroid administration to the dam during the second phase can disrupt normal retinal maturation and result in a thinner than normal retina during late fetal development and at the time of birth (Quinlivan, *et al*., 2000). Cell division within the ovine retina is usually complete by day 125 of gestation, as evidenced by a lack of mitotic figures within the retinal cell population at this point, but lateral expansion of the retina continues beyond this point as the eye increases in size between 120 and 145 days of gestation. Most of the retinal growth which occurs in the sheep after day 125 is due to production of extracellular matrix, cellular processes, synapse formation and dendritic maturation, thereby resulting in expansion of the plexiform layers (Quinlivan, *et al*., 2000). While lateral expansion of the retina accounts for some thinning of the cellular layers in late gestation, there is also a period of apoptosis

of newly formed retinal cells. This occurs as there is an overproduction of neurons in early development, so later culling of excess cells is necessary to attain the number required in adult life; a phenomenon observed in all populations of central nervous system neurons as well as the retina (Stone *et al*., 1999).

Generally, the retinal concentration of the predominant visual pigment, rhodopsin, has a positive correlation with the development of rod outer segments. The eye is capable of detecting and responding to light well before retinal development is complete, as evidenced by the detection of a pupillary light reflex in sheep from 92 days of gestation onwards, when there are only a small number of short photoreceptor outer segments present and the rhodopsin concentration is 0.01 times that of an adult sheep (Höglund, Nilsson, & Schwemer, 1982). Similarly, in advanced cases of retinal degeneration, an animal can be functionally blind, but a pupillary light reflex can still be elicited with bright light if a small number of photoreceptors remain intact (Ofri, 2008c). By 5 days prior to birth, the rhodopsin concentration in fetal sheep increases to 0.6 times that of adult sheep, but the adult concentration of rhodopsin is not reached until 40 days after birth, indicating that photoreceptor development continues postnatally (Höglund, *et al*., 1982). However, all layers of the retina are observable histologically in sheep at the time of birth, in contrast to dogs and cats where significant retinal development occurs after birth, and normal retinal histology is not observed until postnatal day 42 (Aguirre, Rubin, & Bistner, 1972).

1.4 STRUCTURE OF THE RETINA

Common to all vertebrates, the retina of the sheep is inverted. Photoreceptors are located on the outer surface of the retina, away from the pupil through which light enters, and their outer segments face towards the back of the eye. This design is necessitated by the relative membrane instability and rapid turnover of photoreceptor outer segments, meaning that the outer segments need to appose an efficient phagocytic membrane (Stone, *et al*., 1999). The retinal pigmented epithelium (RPE), derived from the outer layer of the posterior optic cup, contains phagosomes which perform this function. Only a very small number of phagosomes are seen during gestation, an observation which may indicate that there is less turnover of photoreceptor outer segments during the fetal period than in adult sheep (Höglund, *et al*., 1982).

The retina has an exceptionally high demand for both glucose and oxygen, meaning that even a very mild change, or compromise in the retinal circulation, can result in ischaemic damage. In most species (including sheep) the retina has a dual blood supply from the choroid and inner retinal vessels (Ofri, 2008b). Photoreceptors have particularly high energy requirements, but the outer retina does not have an intrinsic blood supply and instead relies on the diffusion of nutrients across the RPE from the choroidal circulation. The choriocapillaris is the most permeable capillary bed in the body and blood flows through the choroid rapidly, resulting in delivery of high levels of oxygen and nutrients to the photoreceptors (Stone, *et al*., 1999). The middle and inner layers of the retina are supplied by the retinal vessels which lie in the nerve fibre, ganglion cell and inner plexiform layers (Dubielzig *et al.*, 2010), and these vessels are visible on fundoscopic

examination. Sheep have a holangiotic pattern of inner retinal vasculature, meaning that all quadrants of the retina are vascularised by cilioretinal arteries that emerge around the optic disc and extend to the periphery (Ofri, 2008b). Usually, sheep have 3 or 4 paired arteries and veins in the dorsal, ventral, ventronasal, and ventrotemporal quadrants, along with another five to eight arterioles and venules in association with the nasal and temporal portion of the optic disc (Boileau & Gilmour, 2012). A physiological barrier between the bloodstream and retina (blood-retinal barrier) limits the passage of substances into the retina, and is formed by the endothelial cells and basement membrane of the inner retinal vessels, as well as by the RPE. The blood-retinal barrier prevents large molecules moving between the choroid and retina (Ofri, 2008b).

Before considering the structure of the retina, brief mention needs to be made of the tapetum, which is located superiorly within the inner stromal layer of the choroid (Boileau & Gilmour, 2012). In herbivores such as sheep, the tapetum is fibrous in nature *(tapetum fibrosum)*, whereas in carnivores it is cellular with reflective crystals *(tapetum cellulosum).* The reflective properties of the tapetum, rather than the presence of pigment molecules, are responsible for the distinctive colouration of the fundus (Miller, 2008b), which varies from blue to green to yellow between individuals, but does not appear to change over time (Bellairs, Harkness, & Harkness, 1975). It is generally considered that the function of the tapetum is to reflect light which is not initially absorbed by the photoreceptors back onto the retina, and while it may increase sensitivity of the retina in low light conditions, numerous animals which are largely inactive at night still have a tapetum (such as sheep and dogs). The lower part of the tapetum in sheep and goats lies under an area of increased

ganglion cell density within the retina, and it appears as though this region is parallel to the horizon when the animal is grazing and at rest. This means that light from the earth below the horizon is reflected more than that from the sky above, thereby resulting in increased retinal sensitivity in the region of the visual field that would be most important for the detection of predators. Collagen fibrils within the tapetum are regularly arranged and mostly orientated in one direction; it has been suggested that this may allow differentiation of light polarised in different directions (Bellairs, *et al*., 1975).

Histologically, the retina is composed of ten layers, including the retinal pigment epithelium which is not part of the neurosensory retina. From outer (facing the choroid) to inner (facing the vitreous), the layers are as follows (Ofri, 2008b):

1. Retinal pigment epithelium
2. Photoreceptor layer (rods and cones)
3. External limiting membrane
4. Outer nuclear layer
5. Outer plexiform layer
6. Inner nuclear layer
7. Inner plexiform layer
8. Ganglion cell layer
9. Nerve fibre layer
10. Internal limiting membrane

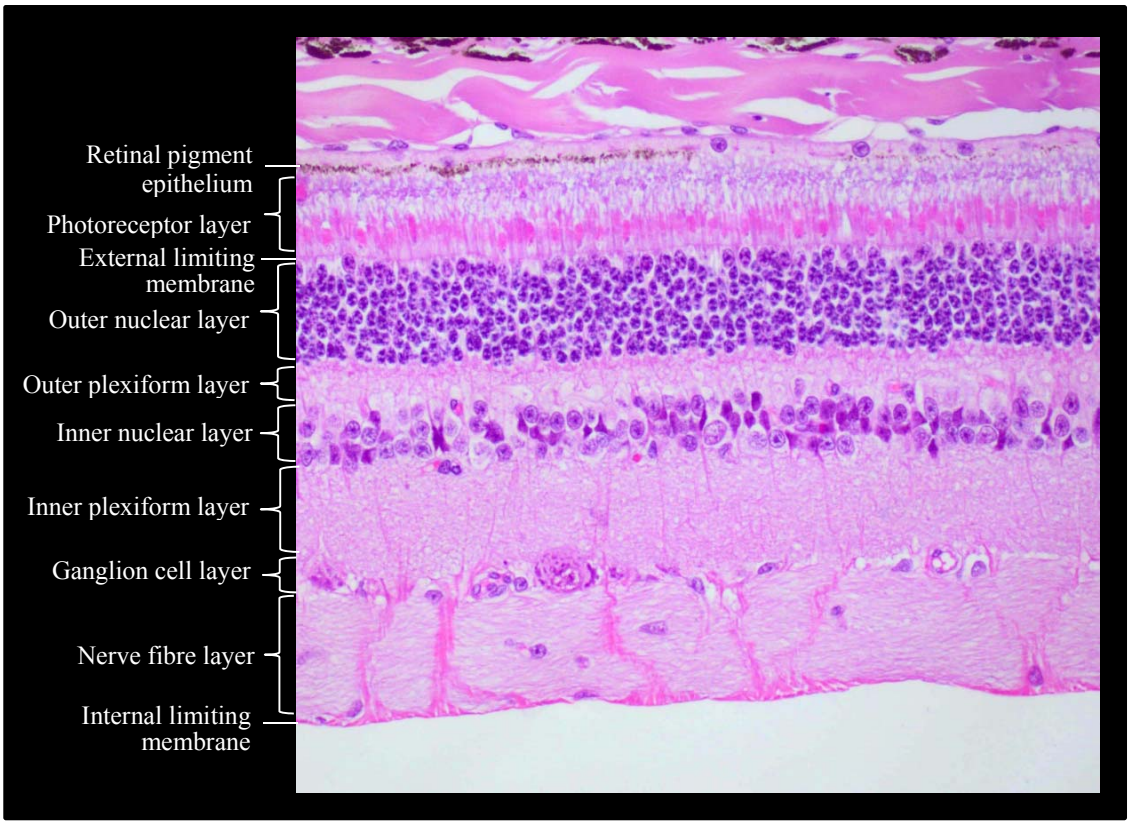

Figure 1.1: **Histological structure of the normal ovine retina** (H&E, 400x). The outer layers of the retina, closest to the back of the eye, are uppermost.

The retinal pigment epithelium (RPE) is composed of a single layer of cuboidal cells that have microvillous processes along the apical surface, which interdigitate with the outer segments of the photoreceptors (de Lahunta & Glass, 2009). In the non-tapetal region, the RPE imparts a brown-black colour to the fundus, but in the tapetal region it is non-pigmented, as melanosomes are lost through autophagocytosis during development (Dubielzig, *et al*., 2010). The RPE has a number of essential roles in the function of the neurosensory retina, including phagocytosis of shed photoreceptor outer segments, uptake and regeneration of retinol (the vitamin A aldehyde essential for vision), and transport of

nutrients and oxygen from the chorocapillaris to the outer retina. Tight junctions between cells of the RPE also form an important part of the blood-retinal barrier, and melanin pigment granules within the RPE help to protect the retina from light damage and oxidative stress (Schraermeyer & Heimann, 2006).

The outermost layer of the neurosensory retina is the photoreceptor layer. Photoreceptors are specialised afferent somatic neurons, the nuclei of which are located in the external nuclear layer. There are two types of photoreceptor cells: rods, which are primarily responsible for vision in dim light; and cones, which function in bright light and are important for colour vision and visual acuity. In sheep and other ungulates rods predominate, with the ratio of rods to cones reported as 30:1 to 40:1 for sheep (Braekevelt, 1983). Two types of cones are present, enabling dichromatic colour vision (Jacobs, Deegan, & Neitz, 1998). Both rods and cones are composed of inner and outer segments, joined by a connecting stalk. The outer segments are derived from highly modified cilia and represent the dendritic zone of the neuron. They are composed of parallel stacked, regularly spaced membranous discs (600-1000 per photoreceptor in people) formed by transverse folding of the plasma membrane. In cone cells, these discs remain continuous with the plasma membrane, whereas those of rods separate soon after they form, making them membranous cytoplasmic compartments (Ross, Kaye, & Pawlina, 2003). Throughout life, the discs are continually produced and migrate outwards, until they are eventually shed and phagocytosed by the RPE. In rods, the discs of the outer segment are of uniform diameter creating a cylindrical shape, and are connected to the elongate inner segments by a narrow neck. Rod outer segments are tall and slender, and measure 40-50µm in length and 2-3µm

in diameter in sheep. Cones, by contrast, have a tapering outer segment and are shorter than rods, with a total length of 30-35μm in sheep (Braekevelt, 1983).

Associated with the membranous discs of photoreceptors are the visual pigment molecules: rhodopsin in rods and iodopsin in cones. Rhodopsin and iodopsin differ in their protein components (known as opsins), giving them different spectral sensitivities; however, they share the same chromophore, 11-*cis*-retinal, which is an aldehyde of vitamin A (Ross, *et al*., 2003). Inadequate dietary vitamin A intake can result in decreased sensitivity to light by photoreceptors, known as night blindness or nyctalopia (Dowling, 2002). The process of vision begins in photoreceptor outer segments, when a photon of light is absorbed by a visual pigment, activating opsin. This in turn activates transducin, and the cascade continues with activation of phosphodiesterase, which then hydrolyses cytoplasmic cGMP. As the concentration of cGMP decreases, cGMP-gated plasma membrane channels close, leading to hyperpolarisation of photoreceptors and an action potential transmitted to downstream neurons (Miyadera, Acland, & Aguirre, 2012).

The inner segments of both rods and cones are also found within the photoreceptor cell layer. These represent the cytoplasmic portion of the cell, and can be divided into ellipsoid (outer) and myoid (inner) parts. The ellipsoid area is rich in mitochrondria, and the myoid portion contains a Golgi apparatus, rough endoplasmic reticulum and ribosomes; as photoreceptor cells are highly metabolically active with significant protein synthesis occurring (Dubielzig, *et al*., 2010). Sheep, like other mammals, have an area centralis in the central retina where cone density is maximal, although even in this area rods outnumber

cones (Ofri, 2008b). Associated with this, there is a single visual streak in sheep, where ganglion cell density is highest, whereas goats have two visual streaks running horizontally and vertically (Boileau & Gilmour, 2012).

Thin outer fibres from the photoreceptor cell processes pass through the external limiting membrane to the photoreceptor cell nuclei, located in the outer nuclear layer. The external limiting membrane is formed by tight junctions between photoreceptor cell membranes and Müller's cells, which are glial cells that span the entire retina (Bringmann & Reichenbach, 2001; de Lahunta & Glass, 2009). In the outer nuclear layer of sheep, there are approximately 15 to 20 rows of photoreceptor cell nuclei, but this thins towards the edge of the retina as photoreceptor density decreases (Dubielzig, *et al*., 2010). Cone nuclei tend to be slightly larger and more vesicular than rod nuclei, and are located adjacent to the external limiting membrane, whereas rod nuclei are present at all levels of the outer nuclear layer (Braekevelt, 1983; Samuelson, 2007).

Adjacent to the outer nuclear layer is the outer plexiform layer, which is formed by synapses between axonal extensions of photoreceptor cells, termed inner fibres, and the dendrites of horizontal and bipolar cells. Using electron microscopy, it has been shown that inner rod fibres have a small knob-shaped synaptic terminal named a spherule, while inner cone fibres have a wider synaptic terminal known as a pedicle (Dubielzig, *et al*., 2010). As illustrated by the photomicrograph in Figure 1.1, the outer plexiform layer is significantly thinner than the inner plexiform layer.

The inner nuclear layer contains the cell bodies and nuclei of four cell types. The outermost nuclei in this layer (along the scleral aspect) belong to horizontal cells, which have large nuclei with a single prominent nucleoli (de Lahunta & Glass, 2009). Horizontal cells are interneurons that connect rod spherules, cone pedicles and bipolar cells. They are thought to enhance contrast between light and dark regions and provide feedback inhibition to photoreceptors (Masland, 2001). In the middle of the inner nuclear layer lie the cell bodies of bipolar cells, whose role is to transmit impulses generated by the photoreceptors to ganglion cells. For this purpose, bipolar cells have processes extending into both the outer and inner plexiform layers and generally form synaptic connections with multiple cells in each layer (Ross, *et al*., 2003). Despite there being many times more rod photoreceptors than cones, the number of cone bipolar cells exceeds the number of rod bipolar cells in most mammals. This is because many rods converge on one bipolar cell to provide increased sensitivity to light, while fewer cones (often only one) synapse on each bipolar cell to provide visual acuity (Masland, 2001). The third type of cell in the inner nuclear layer is the amacrine cell, which is another type of interneuron. Amacrine cell nuclei are located in the inner part of the layer and their processes lie within the inner plexiform layer, where they synapse with bipolar neurons, ganglion neurons and other amacrine cells (de Lahunta & Glass, 2009). The final type of nuclei within the inner nuclear layer is that of the Müller cell. These cells provide structural support to all neural cells through their thin cytoplasmic processes that extend from the external limiting membrane to the internal limiting membrane. Müller cells also perform important metabolic functions (Bringmann & Reichenbach, 2001; de Lahunta & Glass, 2009).

The inner plexiform layer is formed by the cell processes of retinal ganglion cells, bipolar neurons and amacrine cells, and is therefore a site of synaptic interactions. Adjacent to this is the ganglion cell layer, which consists of a single layer of large multipolar neurons, except in the area centralis where there may be 2 or 3 rows of ganglion neurons. These cells have lightly staining nuclei, prominent nucleoli, and Nissl bodies in their cytoplasm (Ross, *et al*., 2003). Also within this layer are retinal blood vessels and small neuroglial cells. The axons of ganglion cells form the nerve fibre layer, which is unmyelinated. At the posterior pole of the eye, these axons coalesce to form the optic disc, which appears white on fundic examination, as the axons become myelinated by oligodendroglia when they penetrate the sclera at the *area cribosa* (de Lahunta & Glass, 2009). In sheep, the optic disc is kidney shaped (Maggs, 2008). The final layer of the retina is the inner limiting membrane adjacent to the vitreous; this is a basement membrane formed by the terminal processes of Müller cells.

In sheep, there is more than 80% decussation of fibres at the optic chiasm, i.e. less than 20% of fibres remain ipsilateral, the rest cross to the contralateral side to form the optic tracts (Boileau & Gilmour, 2012). The optic tracts pass laterally from the chiasm and course ventral to the cerebral peduncle, before curving dorsally between the peduncle and pyriform lobe. From these tracts, 20-30% of the fibres branch off to enter the pretectal area, where they participate in the pupillary light reflex. The rest of the fibres continue on to the lateral geniculate nucleus where they synapse with ascending neurons. From here, fibres pass forward as the optic radiation, which enters the visual cortex where interpretation

occurs. In addition, subcortical integration of vision is thought to occur at the superior colliculus (Miller, 2008a).

1.5 RETINAL DISEASE IN SHEEP

There are a variety of causes of retinal disease in sheep, including toxic, infectious, dietary and inherited aetiologies. Terminology used to describe different disease processes is often used interchangeably, but in this discussion the nomenclature used will be based on the paper by Warburg & Moller (1989). Degeneration is a broad term implying loss of function, and does not distinguish between genetic and acquired aetiologies. To be more specific, the terms *dystrophy* and *retinopathy* can be used. Dystrophy denotes the process and consequences of a hereditary progressive disease, in which affected tissues initially have normal function and the time on onset can be variable. For example, in the retina there can be rod dystrophies, cone dystrophies or rod/cone dystrophies, and individuals with these dystrophies are normal at birth, but vision deteriorates over time. Acquired lesions, such as toxic and inflammatory processes, are not dystrophies and are instead referred to as retinopathies. An additional term, *dysplasia*, is used to describe developmental abnormalities of tissue structure, where the abnormality is present at birth and does not progress over time.

1.5.1 TOXIC RETINOPATHIES

One of the earliest described retinopathies in sheep is bright blindness, a progressive retinal degeneration first recognised in hill flocks on the North Yorkshire moors in the United Kingdom (Watson, Barlow, & Barnett, 1965). Affected sheep are usually 3 to 4

years of age, and on ophthalmologic examination have poorly responsive pupils, narrowing of retinal blood vessels and increased tapetal reflectivity. Histologically, fragmentation of the outer segments of rods and cones is seen and there is swelling of proximal segments of photoreceptors, mainly cones. As the disease progresses, the rods and cones, along with the outer nuclear layer and parts of the inner nuclear layer, are completely destroyed and the pigment epithelium lies adjacent to the remainder of the inner nuclear layer (Watson, Barnett, & Terlecki, 1972). Bright blindness is due to chronic ingestion of bracken fern, which contains the toxic principle 'ptaquiloside' (Hirono *et al.*, 1993), although the exact pathogenesis of the disease is unclear. Proposed mechanisms include either altered retinal circulation due to narrowing of blood vessels, or decreased retinal lactate dehydrogenase activity (Sweasey, Patterson, & Terlecki, 1971; Hirono, *et al.*, 1993). The bracken species present in New Zealand do contain ptaquiloside (Smith *et al*., 1988), but bright blindness has not been reported in this country (Smith, 1990).

Numerous other plant species have also been implicated in the development of blindness in sheep. In sheep and goats in southern Africa, particularly Nambia, a degenerative retinopathy has been reported due to the ingestion of *Helichrysum argyrosphaerum* (Lugt, Olivier, & Jordain, 1996). Lesions associated with this plant include loss of photoreceptor outer segments and subsequent hyperplasia and hypertrophy of the retinal pigment epithelium, focal retinal detachment and thinning or loss of nuclear layers. In addition, the toxic principle causes an optic neuropathy, myelin oedema and status spongiosus (myelin vacuolation) in the white matter of the brain, giving rise to blindness, paresis and paralysis. In Western Australia, 'blindgrass' (*Stypandra imbricate*)

can cause permanent blindness, if animals do not die of acute toxicity (Main *et al*., 1981). Similar to other toxic retinopathies described, the lesions of blindgrass poisoning include loss of photoreceptors and outer nuclear layers of the retina, degeneration and sclerosis of the optic nerve (optic neuropathy) and status spongiosus of cerebral white matter. A single toxic dose is thought to be responsible in most reported cases, with lesions becoming apparent several weeks after exposure.

Locoweeds (*Astragalus* and *Oxytropis* spp.) contain swainsonine, and toxicity is reported to cause apparent blindness in sheep and cattle (James, 1989). Descriptions of associated ocular pathology are limited, but marked cytoplasmic vacuolation of ganglion cells and bipolar neurons in the retina occurs with experimentally induced locoweed poisoning. There is also hypertrophy and vacuolation of the epithelium covering the ciliary body, but the remainder of the eye, including the optic nerve, is unaffected (Van Kampen & James, 1971).

In addition to plant toxicities, certain synthetic compounds may also cause retinopathies. The best example in sheep is that of closantel, a halogenated salicylanilide anthelmintic which is used for the control of *Haemonchus* spp., *Fasciola* spp. and *Oestrus ovis* (Swan, 1999) . Clinically, overdosage of closantel is associated with ataxia, paresis, recumbency and blindness with mydriasis and papilloedema. Acutely in the retina, there is necrosis of the outer layers and focal areas of haemorrhage with a cellular exudate in the subretinal space. More chronic cases have a single layer of nuclei remaining in the outer nuclear layer, with a small number of tiny, blunt processes in the photoreceptor layer,

thought to be surviving cones (Van der Lugt & Venter, 2007). This may suggest that rods are selectively targeted in closantel toxicity, but the mechanism of toxicity has not been elucidated. Clostantel toxicity also causes status spongiosus of the white matter in the brain and spinal cord, and myelin oedema of the optic nerve (Gill *et al*., 1999; Van der Lugt & Venter, 2007). The initial myelin oedema causes swelling and compression of the nerve within the optic canal, and chronically this leads to narrowing of this portion of the nerve due to necrosis and fibrosis.

It is worth noting that the retinal changes described in the various toxicities listed here represent direct retinotoxic effects, as retrograde degeneration of the photoreceptor layer is not seen with optic nerve damage, although the ganglion cell layer will be affected over time (Spencer, 1985). However, damage to the retina and optic nerve may share a common toxic mechanism in some instances.

1.5.2 INFECTIOUS RETINOPATHIES

A variety of infectious organisms are capable of causing inflammatory retinopathies or retinitis in sheep. The choroid and the retina are in close association, therefore inflammation from the choroid will often spread to the retina, which is termed chorioretinitis, or from the retina to the choroid, termed retinochoroiditis (Ofri, 2008b). An example of retinochoroiditis is ocular toxoplasmosis, caused by the ubiquitous intracellular protozoan *Toxoplasma gondii*, of which the cat is the definitive host (Rothova, 1993). Ocular toxoplasmosis is much less common than other manifestations of infection, but the organism can cause focal necrosis of the retina, along with uveitis, and the inflammatory

infiltrate associated with this in sheep is predominantly composed of epithelioid macrophages (Piper, Cole, & Shadduck, 1970). In humans, ocular toxoplasmosis can result from congenital or postnatal infection, and can be responsible for recurring episodes of retinal damage and subsequent retinochoroidal scarring. During active episodes, viable proliferating parasites are present within the retinal lesions (Holland, 2003). Another protozoan, *Trypanosoma brucei,* which lives in the bloodstream, has been reported to cause retinitis in humans with mononuclear vascular cuffs around retinal blood vessels, loss of cells in the ganglion layer and optic neuritis, however the lesions in the anterior chamber are normally more remarkable (Ikede, 1974).

While a number of bacterial organisms are capable of causing keratitis, conjunctivitis and uveitis, bacteria are not usually associated with primary retinal disease in sheep. However, bacterial septicaemia, especially in young animals, may cause chorioretinitis as well as uveitis, potentially leading to scarring of the retina (Aroch, Ofri, & Sutton, 2008). These scars appear as hyperreflective areas in the tapetum which may be hyperpigmented centrally, while in the non-tapetal region they appear as pigmented grey lesions. Blindness is not usually a feature.

In cattle, fetuses infected with bovine viral diarrhoea virus (BVD) between 79 and 150 days of gestation may exhibit retinal atrophy, optic neuritis, microphthalmia with retinal dysplasia and cataracts, and cerebellar hypoplasia (Bistner, Rubin, & Saunders, 1970; Wilcock, 2007). Similarly, the pestivirus of sheep, Border Disease Virus, can also cause congenital defects when infection occurs between 16 and 90 days of gestation.

However, lesions of *in-utero* infection are usually confined to the CNS, skeletal, integumentary and endocrine systems, and no specific retinal lesions have been reported (Sawyer, 1992). A virus which can affect the retina in fetal sheep is Bluetongue virus, which is arthropod-borne and causes a vasculitis, with specific strains capable of transplacental transmission and fetal infection (Maclachlan *et al.*, 2009). Lambs born to ewes naturally infected or vaccinated with a modified live vaccine during the first half of pregnancy (particularly day 50-55 of gestation) develop a necrotising retinopathy and retinal dysplasia, along with central nervous system malformations including hydrocephalus and cerebellar hypoplasia (Osburn, 1972; Boileau & Gilmour, 2012). Bluetongue virus is not currently present in New Zealand.

Scrapie, a transmissible spongiform encephalopathy of sheep and goats, is associated with slowly progressive central nervous system disease, and abnormal prion protein (PrP^{Sc}) may accumulate in the retina. Immunoreactivity for PrP^{Sc} is predominantly present in the inner and outer plexiform layers of the retina, and is associated with GFAP immunoreactivity, which can indicate retinal stress (Greenlee, Hamir, & Greenlee, 2006). Accumulation of PrP^{Sc} may not be accompanied by histological evidence of retinal degeneration, or prion protein may be visible as a granular deposit in the inner plexiform and ganglion cell layers. In severely affected sheep, both nuclear layers may also be affected and become atrophic, while Müller cells become hypertrophic (Hortells *et al*., 2006). Lesions are rarely detected in photoreceptor outer segments. There is also accumulation of PrP^{Sc} and spongiosis within the visual tracts of the brain during the preclinical stage. This disease is not currently present in New Zealand.

In sheep in North America, infection with the nematode *Elaeophora schneideri* has been reported to cause retinal disease, characterised by chorioretinal atrophy with increased tapetal pigmentation, attenuation of retinal vasculature, and optic nerve atrophy (Abdelbaki & Davis, 1972). Parasites may be identifiable on ophthalmic examination, but affected sheep are not usually blind. Adult *E. schneideri* nematodes are usually found in the carotid arteries. Mule deer and black-tailed deer are considered to be the normal definitive hosts and do not exhibit clinical signs of infection (Boyce *et al*., 1999).

In other species, particularly farm dogs in New Zealand, aberrant migration of nematode larvae can cause multifocal retinitis and retinal atrophy. This disease is known as ocular larva migrans and *Toxocara* sp. have been implicated (Hughes, Dubielzig, & Kazacos, 1987).

1.5.3 NUTRITIONAL DEFICIENCIES

Vitamin A is a fat soluble vitamin which has numerous roles in the body, including in vision. As described earlier, there are two types of visual pigment, rhodopsin in rod cells and iodopsin in cones. Each of these is composed of a protein opsin and a chromophore. The chromophore of rhodopsin in all vertebrate species is 11-*cis*-retinal, which is formed from all-*trans*-retinol, otherwise known as the active form of vitamin A (Crouch *et al.*, 1996). Absorption of a light photon causes *cis*-*trans* isomerisation of 11-*cis*-retinal, and the isomerised retinal is able to trigger the GMP cascade of phototransduction, ultimately resulting in the generation of an action potential (Pepe, 1999). The isomerised retinal is then recycled via the retinoid cycle in the retinal pigment epithelium (Miyadera, *et al*.,

2012). Therefore, the process of phototransduction is dependent on vitamin A, and hypovitaminosis A results in impaired rod function and night blindness in numerous species (Moore, 1939; Dowling & Wald, 1960). Ruminants are able to efficiently convert beta-carotene from pasture into vitamin A, but deficiency can occur if they are fed high grain diets, or forages such as hay that have been stored over a long period (Commonwealth Scientific and Industrial Research Organisation (CSIRO), 2007). In addition, vitamin A uptake may also be impaired by severe endoparasitism. Large reserves of vitamin A can be maintained in the liver in the form of retinol, so sheep may not show clinical signs of deficiency, even after a long period of reduced vitamin A intake, such as during a drought (Gartner, 1969). Ruminants that develop night blindness as a consequence of vitamin A deficiency generally have dilated, unresponsive pupils, with papilloedema, retinal haemorrhages and depigmentation of the non-tapetal retina on fundic examination (Eveleth, Bolin, & Goldsby, 1949; Paulsen *et al*., 1989). In most cases, the night blindness resolves with vitamin A supplementation.

Other dietary components, such as the amino acid taurine, are also present in the retina and may be important in vision (Lombardini, 1991). For example, the amino acid taurine is thought to protect photoreceptors from light and chemical damage, and regulate calcium transport and signal transduction. In cats and some primates, taurine deficiency can lead to central retinal degeneration, but sheep are able to synthesise taurine from endogenous precursors, thus a deficient diet has no effect on vision.

1.5.4 INHERITED RETINAL DISEASES IN SHEEP

Inherited retinal diseases are rarely described in sheep. Of those that are recognised, most also affect other parts of the eye or the central nervous system, as is seen in congenital microphthalmia (De Groot, 1957) and ceroid lipofuscinosis (Jolly *et al.*, 1980). To the author's knowledge, the only inherited primary retinal disease reported in sheep is a day blindness in Awassi sheep, associated with cone dysfunction (Shamir *et al*., 2010).

1.5.4.1 Day blindness in Awassi sheep

An inherited day blindness occurs in improved Awassi lambs in Israel, and is characterised by diminished cone function on electroretinographic examination. Visual impairment is detectable as early as 1 day of age in affected lambs, and visual performance is the same in both dim and bright light. Normal retinal histology is preserved in this disease, and large numbers of both red-green and blue cones are observed in affected sheep using immunofluorescence (Shamir, *et al*., 2010). These findings are similar to achromatopsia in human beings, a type of stationary cone dystrophy. The day blindness is associated with a stop codon mutation in the cone photoreceptor cyclic nucleotide-gated (CNG) channel subunit α gene, and all affected animals tested were homozygous for this mutation (Reicher, Seroussi, & Gootwine, 2010), supporting an autosomal recessive mode of inheritance. Identification of the mutation has allowed ram culling strategies to be applied at Jordanian Awassi Breeding Stations in order to facilitate gradual eradication of the trait (Jawasreh *et al.*, 2012).

1.5.4.2 Congenital microphthalmia in Texel sheep

Retinal dysplasia is a feature of congenital microphthalmia in the Texel breed (Hanset, 1961). In this disease, the globe is approximately half the normal size; all embryonic components of the eye are present but are dysplastic. Histologically, the retinas of affected sheep have identifiable inner and outer nuclear layers, but additional rosettes of cells from the nuclear layers are seen deeper in the retina. The normal contact between the RPE and sensory retina is not observed, ganglion cells are reduced in number and the optic nerve is hypoplastic (Roe *et al.*, 2003). Microphthalmia causes complete blindness, but there are no abnormalities in other tissues. The disease is inherited in an autosomal recessive manner and is associated with a missense mutation in the *PITX3* gene, which encodes a homeodomain-containing transcription factor important in lens formation (Becker, *et al.*, 2010).

1.5.4.3 Other inherited ocular diseases in sheep

Other inherited ocular diseases have been described in sheep, but in these diseases the retina is unaffected. An example is inherited cataracts in New Zealand Romney sheep (Brooks *et al.*, 1982). These sheep develop bilateral cortical cataracts from 1 to 2 months of age, and total lens opacity occurs at 10 to 11 months old. The disease is inherited in an autosomal dominant manner and pathological changes appear confined to the lens, with no involvement of the retina. A specific causative mutation has not yet been identified, but recent genome mapping indicates a region of ovine chromosome 6 is strongly linked to the development of cataracts in these sheep (Wilson *et al*., 2012).

1.5.4.4 Retinal abnormalities with ceroid lipofuscinoses in sheep

Retinal abnormalities may also occur in association with generalised heritable diseases. In sheep, for example, the neuronal ceroid lipofuscinoses (NCLs) lead to secondary retinal dystrophy and atrophy. The NCLs are a group of recessively inherited lysosomal storage diseases that cause progressive neurodegeneration, characterised clinically by deteriorating mental and motor function, blindness and premature death. Currently, human NCLs are classified into ten types (CLN1 to CLN10) based on the gene responsible, but there is some phenotypic variation within each type as different mutations are possible in a given gene, and not all genetic associations have been identified to date (Mole, Williams, & Goebel, 2011). This nomenclature is also applied in animals, and in sheep CLN5 and CLN6 have been well described, including the associated retinal pathology. All forms of ceroid lipofuscinosis have two common features; progressive atrophy of the cerebral cortex, and accumulation of fluorescent storage bodies in neurons and most cells throughout the body (Jolly*, et al.*, 1980; Jolly *et al.*, 2002; Tammen *et al.*, 2006).

CLN5 has been identified in a flock of New Zealand Borderdale sheep (Jolly*, et al.*, 2002) and in Australian Devon cattle (Harper *et al*., 1988). In both sheep and cattle, blindness develops from 14 to 15 months of age and retinal pathology is characterised by almost complete loss of both rod and cone photoreceptors, resulting in a non-existent outer nuclear layer. Less severe loss of nuclei occurs in the inner nuclear layer and ganglion cell layer, and remaining retinal cells contain storage granules (Harper *et al.*, 1988; Jolly *et al.*, 1992).

CLN6 occurs in New Zealand South Hampshire sheep, and has been extensively researched as a model for the analogous human disease. Affected sheep are normal at birth, but begin to show subtle evidence of neurological disease at 10 to 14 months of age, and blindness typically develops early in the clinical course of the disease (Jolly & West, 1976; Jolly*, et al.*, 1980). The blindness has both a central component, due to atrophy of the cerebral cortex, and a peripheral component, resulting from retinal dystrophy (Graydon & Jolly, 1984; Mayhew *et al.*, 1985). Retinal lesions include the formation of dystrophic and shortened outer segments of rod photoreceptors, with subsequent shortening of cone outer segments. Over time, there is also progressive loss of nuclei within the outer nuclear layer of the retina. CLN6 has also been diagnosed in a flock of Australian Merino sheep (Cook *et al*., 2002; Tammen, *et al*., 2006).

1.6 INHERITED RETINAL DISEASES IN OTHER SPECIES

In human beings, there are a large number of inherited, progressive retinal disorders that are clinically recognised as 'retinitis pigmentosa'. Similarly in dogs, many retinal dysplasias and degenerations are well described in a number of breeds, and are collectively termed canine generalised progressive retinal atrophy (Clements *et al.*, 1996).

1.6.1 RETINITIS PIGMENTOSA IN HUMANS

Retinitis pigmentosa (RP) is a term used to encompass a range of retinal dystrophies characterised by primary degeneration of rod photoreceptors (Voaden, 1991). Inheritance can be autosomal dominant (accounting for approximately 30-40% of cases), autosomal recessive (50-60% of cases) or X-linked (5-15%), although rarer forms with non-Mendelian

inheritance do exist (Hartong, Berson, & Dryja, 2006). The majority of cases are confined to the eye and are referred to as simple, or non-syndromic RP, in order to distinguish them from syndromic RP, in which other organs are also affected, and systemic RP, where the retinal disease is secondary to systemic pathology (Ferrari *et al*., 2011). Syndromic and systemic RP together represent approximately 25% of all retinitis pigmentosa cases (Daiger, Bowne, & Sullivan, 2007).

The most common clinical history in cases of retinitis pigmentosa is the onset of night blindness during adolescence, with loss of the mid-peripheral visual field. Histologically, this correlates to shortening of the outer segments of rod photoreceptors. The condition is progressive, and over time far peripheral vision is lost, leading to tunnel vision, with most patients considered legally blind by 40 years of age. Eventually, central vision is also lost, usually by 60 years of age. On fundic examination, features of retinal degeneration can be identified, especially attenuation of retinal blood vessels. The optic disc often appears pale and waxy, and there may be pigment deposits in the retina where epithelial cells of the RPE have migrated into spaces left by photoreceptor death, thus giving the disease its name (Milam, Li, & Fariss, 1998; Hartong*, et al.*, 2006)

Retinitis pigmentosa is a genetically heterogenous condition, and to date a total of 50 genes and loci have been identified as causing non-syndromic RP; 19 of which correspond to autosomal dominant forms, 26 to autosomal recessive forms and 5 to X-linked inheritance (Daiger, 2012). Additional genes and loci are associated with syndromic and systemic forms of RP. However, only approximately 60% of retinitis pigmentosa cases are

accounted for by the mutations currently discovered, with the molecular basis of the remaining 40% of cases currently unknown (Anasagasti *et al.*, 2012). A table of genes involved in retinitis pigmentosa, modified from the seminar paper by Hartong, *et al*. (2006), is included in the Appendix (Table A.1.) of this thesis.

1.6.2 RETINAL DEGENERATIONS IN DOGS

A large number of inherited retinal diseases have been described in dogs, and these can be classified into progressive, stationary and developmental disorders (Miyadera, *et al*., 2012). Progressive retinal atrophy (PRA) is homologous to RP in humans, and is characterised by primary degeneration of rod photoreceptors, leading to the development of night blindness (Clements, *et al*., 1996). Cones photoreceptors may also be affected, but tend to be lost later in the course of the disease. The age of onset, rate of disease progression, and mode of inheritance of PRA in dogs varies greatly, depending on the breed and the mutation responsible.

Fundic examination findings in dogs with PRA are similar to those described in humans with RP and other forms of retinal degeneration, and include progressive attenuation of retinal blood vessels and a paler than normal optic disc. As dogs have a tapetum, there is the additional finding of tapetal hyperreflectivity due to the retina absorbing less light as it becomes thinner, and therefore reflecting more light back to the observer (Ofri, 2008b). A high proportion of dogs with an inherited retinopathy also have cataracts, and it is not known if there is any link between the two diseases or if they occur independently (Adkins & Hendrix, 2005; Ofri, 2008b).

In addition to PRA, there is also a poorly defined retinal degeneration in dogs referred to as 'Sudden Acquired Retinal Degeneration' or SARD, but this is does not appear to be inherited. In this disease, photoreceptor cells undergo apoptosis, and affected dogs present with acute blindness (Miller *et al.*, 1998). The cause of SARD is not well understood, although it has been suggested that an underlying endocrinopathy or autoimmune disorder may be responsible (Ofri, 2008b).

1.6.3 INHERITED RETINOPATHIES IN OTHER SPECIES

1.6.3.1 Cats

Progressive retinal atrophy has been reported in cats, and has been shown to have a hereditary basis in the Abyssinian breed. Two forms of PRA are reported in Abyssinians: a progressive rod-cone degeneration with autosomal recessive inheritance, and a rod-cone dysplasia with earlier onset and autosomal dominant inheritance (Narfström, 1999).

1.6.3.2 Cattle

Primary inherited retinal dysplasia or dystrophy is not reported in cattle. Retinal degeneration was described in a number of Friesian cows within a single herd in the East Midlands in England, but the authors did not establish a hereditary basis for this disease (Bradley, Terlecki, & Clegg, 1982). In these animals, there was thinning of the retina due to loss of photoreceptor cells, and in end-stage disease rods were completely lost, while stumps of cone inner segments remained. Toxic and infectious causes of retinal degeneration were ruled out, but pedigree analysis did not support a disease with simple Mendelian inheritance.

As in other species, a number of inherited storage diseases can affect the retina in cattle, but the disease is not confined to the eye as storage products also accumulate in the central nervous system and other tissues. Examples of storage diseases with retinal involvement in cattle include GM_1 gangliosidosis (Sheahan, Donnelly, & Grimes, 1978), mannosidosis (Jolly *et al.*, 1987) and ceroid lipofuscinosis (Jolly, *et al*., 1992). A detailed discussion of these diseases is beyond the scope of this review.

1.6.3.2 Rodents

There are a number of rodent models of retinal degeneration, with the most common being the rds (retinal degeneration slow) mouse. These mice have a defective *RDS* (peripherin) gene, and this gene is important in the development and maintenance of photoreceptor outer segments. Mice homozygous for a mutation in RDS develop no outer segments, while heterozygotes have shortened, disorganised outer segments (Van Soest *et al.*, 1999). Other rodent models of retinitis pigmentosa include transgenic mice with mutations in the rhodopsin gene (Olsson *et al*., 1992), the cGMP phosphodiesterase genes (Tsang *et al*., 1996), the *RPGR* gene (Hong *et al*., 2000), and the *RPE65* gene (Redmond *et al.*, 1998; Dalke & Graw, 2005). Much of the current research regarding the pathogenesis and treatment of retinitis pigmentosa is conducted in mouse models of the disease.

1.7 AIMS OF THIS THESIS

During 2011 and 2012, several cases of adult-onset blindness were identified in a breeding flock of 140 Wiltshire sheep in Darfield, west of Christchurch. These sheep showed no signs of visual impairment until approximately 2 to 3 years of age, when blindness became noticeable in low light conditions. Over time, the disease in these sheep progressed to complete blindness in the absence of any other clinical signs. Initial investigations revealed the blindness was due to retinal degeneration, with loss of the photoreceptor layer, particularly rod photoreceptors, and attenuation of the outer nuclear layer of the retina. There was no evidence of a toxic cause of retinal degeneration, and a genetic aetiology was considered likely.

The primary objectives of this study were to:

a) Develop a practical method to screen the entire flock (140 sheep) for blindness
b) Describe the phenotypic characteristics of this disease, using fundic examination and histopathology.
c) Determine whether this disease in Wiltshire sheep is inherited, and if so, investigate possible candidate genes using PCR and sequencing techniques.

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Chapter 2

HISTORY, CLINICAL SIGNS & PATHOLOGY OF BLINDNESS IN WILTSHIRE SHEEP

2.1 INTRODUCTION

In 2011 and 2012, adult-onset blindness was identified in a breeding flock of Wiltshire sheep in Darfield, west of Christchurch. A total of nine blind ewes, from a flock of 140 sheep, were identified over a 2 year period. Affected ewes appeared to have normal vision until 2 to 3 years of age, when visual impairment became noticeable in low light conditions. Over several months this usually progressed to complete blindness, as evidenced by an inability to negotiate gateways during mustering in full daylight. No blind rams were identified during this period, but this was considered to be a consequence of rams being sold at a young age, rather than a true sex predilection. Initial clinical examinations of affected sheep revealed that abnormalities appeared to be confined to the

eyes, with no evidence of central nervous system or systemic involvement, and ophthalmic examination findings suggested retinal atrophy was a feature of the disease.

The aim of this study was to describe the clinical signs of adult-onset blindness in Wiltshire sheep and develop an efficient method of screening other sheep in the flock for visual impairment. In addition, the study aimed to provide an insight into disease progression by comparing the appearance of the fundus and retinal histopathology in sheep with early and end-stage disease.

2.2 MATERIALS AND METHODS

2.2.1 EVALUATION OF VISUAL IMPAIRMENT IN SHEEP

Visits to the affected property were undertaken in June and December 2012. On these occasions, an outdoor maze was used to identify visually impaired sheep (Figure 2.1). Maze testing was performed at dusk in low ambient light conditions over several evenings, as prior observations suggested that scotopic (low light) vision was lost prior to photopic (bright light) vision in affected animals. In order to test all sheep under similar light intensities, groups of 3 sheep were run through the maze at the same time. Sheep performance through the maze was assessed by both the breeder and primary investigators, and all sheep on the farm at the time were subjected to maze testing. Any which showed slight hesitation or completed the maze more slowly than their cohorts were drafted off for individual assessment and fundic examination.

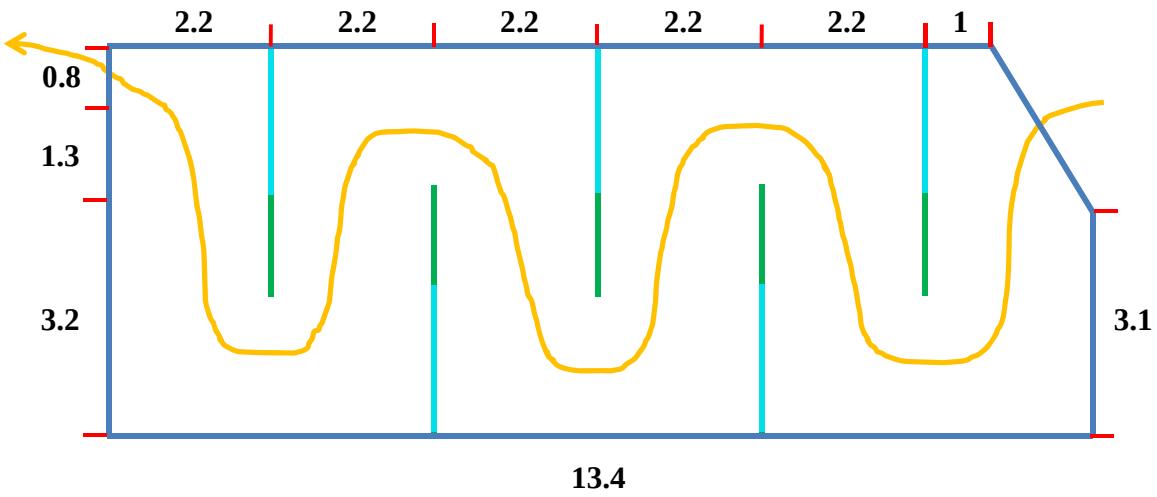

Figure 2.1: **Schematic diagram of maze used to detect visual impairment in sheep.** All measurements are in metres, and indicate the distance between adjacent red lines. The path taken by sheep through the maze is indicated by the yellow line. Barriers within the maze are formed by 1 metre high metal gates (—) and plastic mesh fencing (—).

2.2.2 ANIMALS USED AND SAMPLES COLLECTED

Ophthalmic examinations were conducted in a darkened indoor holding pen on the farm, using a binocular indirect ophthalmoscope (Welch Allyn Inc., USA) and a Pan Retinal 2.2 lens (Volk Optical Inc., USA). Observations were recorded manually by the primary investigator and a certified veterinary ophthalmologist, Steve Heap (BVSc, CertVOphthal). Following the examination, a series of retinal images were obtained from each sheep using a handheld digital fundus camera (ClearView^{TM} Optical Imaging System, Optibrand, Colorado, USA). In some sheep, mild pupillary constriction limited the quality of retinal images captured, and in these cases 1 or 2 drops of tropicamide (Mydriacyl® 1.0%, Alcon Laboratories, USA) was applied to the corneal surface of each eye to facilitate pupillary dilation. A heparinized blood sample was collected via jugular venipuncture from each sheep that underwent ophthalmic examination.

2.2.3 TISSUE PROCESSING AND STAINING

Ocular tissue samples were collected from two blind ewes (65-10 and 43-06) that were being killed by the farmer for home consumption. The eyes were enucleated shortly following death using a transconjunctival approach, and the posterior pole of each eye was separated from the anterior portion using the technique of frontal blocking, as described by Ramos, Reilly & Bolon (2011). With this technique, a cross sectional incision was made a few millimetres behind the limbus, posterior to the lens, using a Thomas blade. The vitreous humor was then removed using a pipette, and the posterior part of one eye from each sheep was immersed in Bouin's solution. After 36 hours in Bouin's solution, each sample was washed with water and placed in 10% formalin for transport to Palmerston North. The contralateral eye of each sheep was also sectioned via frontal blocking and a small piece of the retina was removed and placed in RNAlater RNA Stabilisation Reagent (Qiagen N.V., Netherlands) to be frozen for future use if required. The remainder of the posterior globe was placed in 10% formalin (Gribbles Veterinary Pathology, Christchurch). The head of each sheep was sectioned sagitally to facilitate brain removal, and the brains placed in 10% formalin.

The posterior globes fixed in Bouin's from each sheep were subsequently dehydrated in graded alcohol and embedded whole in paraffin wax. These were then trimmed to the size of a standard tissue cassette using a 6-inch mini hacksaw, and the smaller sections were each re-embedded in paraffin. From these blocks, sections 3 µm thick were cut and stained with haematoxylin and eosin (H & E).

2.3 RESULTS

2.3.1 FARMING PRACTICES

The farming operation was established in 2002, and is based around a flock of 140 Wiltshire ewes. The primary objective of the operation is to produce Wiltshire ewes to sell to other farms, where they are used as breeding stock. Rams are also sold for use as breeding sires on other farms, but most of the ram lambs are sent for slaughter each year. Sheep on the farm are exclusively pasture fed, with no supplements brought onto the property. Silage is made each year in spring and fed out in winter, or in other periods of poor pasture growth such as droughts. Generally, ewes are grazed in mobs of 30-40, except during heavy snowfall when they are set stocked. Paddocks are well fenced, and sheep do not have any access to any non-pasture plant species. No bracken fern is present on the property.

No routine anthelmintic treatments are administered to sheep on the farm. Ram lambs which are perceived to be growing poorly are occasionally given a single dose of a combination oxfendazole / levamisole short-acting oral drench (SCANDA®, Coopers Animal Health Ltd., New Zealand) at recommended dose rates. New sheep entering the farm are drenched with an ivermectin-based product before joining the flock, but ewes born on the property are never drenched. All lambs are vaccinated with a 5-in-1 clostridial vaccine (Ultravac^{TM} 5-in-1, Zoetis New Zealand Ltd., New Zealand), and hoggets are vaccinated against campylobacter (Campylovexin®, Virbac Animal Health, New Zealand). No other animal health products are routinely used.

2.3.2 CLINICAL FEATURES

Four of the nine affected ewes had been culled prior to the start of this investigation, and retinal degeneration in these sheep had been confirmed on fundic examination and retinal histology. All five of the affected sheep remaining on farm appeared normal when observed in the paddock, and were judged to be in similar body condition to unaffected sheep. There was no apparent difference in lambing rates between affected and unaffected ewes, and affected ewes were able to successfully rear twin, and occasionally triplet, lambs. The breeder had observed that, when moving ewes and their lambs, affected ewes tended to follow their lambs through gateways, instead of the ewe leading the lambs as expected. No affected sheep died due to misadventure.

During mustering, affected sheep were often in the middle or towards the back of the mob being moved, and did not show any overt signs of visual impairment while surrounded by other sheep. However, when separated from the flock, three of the affected sheep (43-06, 63-08 and 111-08) appeared hesitant in their movements, particularly in confined spaces such as yards and in low ambient light conditions, and often followed fence lines when moving, or misjudged gateways. The two youngest affected sheep, 102-09 and 65-10, did not show any clinical signs while being mustered or yarded.

2.3.3 EVALUATION OF VISUAL IMPAIRMENT IN SHEEP

All adult sheep on the farm passed through the maze at dusk at least once, and maze testing successfully identified two sheep that had not previously shown any signs of visual impairment. Normal sheep were able to negotiate the maze rapidly and avoid all barriers,

while affected sheep only reached the first or second barrier before becoming disorientated and stopping. Even when affected sheep closely followed normal sheep, they were unable to complete the maze in low light conditions and had to be manually guided to the end by an investigator. The two youngest affected sheep, 102-09 and 65-10, were subsequently able to complete the maze in bright light, but in a slower time than their normal cohorts. Ophthalmological examination confirmed the presence of retinal changes in all sheep identified as affected on the basis of maze testing.

Normal sheep manifested a learning ability, as evidenced by faster completion of the maze on any subsequent repetitions. Affected sheep did not show a similar improvement with repetition, and were unable to complete the maze on subsequent attempts.

2.3.4 OPHTHALMIC EXAMINATION

In total, ophthalmic examinations were performed on 13 sheep in June 2012 by the primary investigator. Of these, three adult sheep were determined to be blind based on retinal appearance, and one sheep had findings suggestive of early retinal disease. The rest of the sheep examined were normal, including three daughters of blind ewes. In December 2012, a certified veterinary ophthalmologist, Steve Heap (BVSc, CertVOphthal) examined the four sheep with previously identified ophthalmic examination abnormalities, as well an additional ewe with visual impairment detected during maze testing in December. Three normal sheep were also examined at this time, including a son of a blind ewe, giving a total of eight animals examined.

All sheep had normal mentation and ambulation, and physical examination findings were unremarkable. On ophthalmic examination, the five sheep that failed to complete the maze test were noted to have dilated pupils, with poor pupillary constriction compared to control sheep. No abnormalities were observed in the cornea, anterior chamber or lens of any sheep, other than a small cataract in the right eye of 63-08, which would not significantly impair vision. All affected sheep had significant, bilateral retinal changes on fundic examination, and in general the severity of these changes worsened with advancing age. The most striking feature was attenuation of retinal blood vessels, which was evident in even the youngest of the affected sheep (2 years of age). Thinning of the dorsal cilioretinal arterioles and venules, along with their smaller tributaries, was easily appreciable, as is demonstrated in Figure 2.2. In addition to blood vessel changes, all affected sheep exhibited generalized patchy tapetal hyperreflectivity which radiated laterally, with large areas of the tapetum appearing brighter than usual. The oldest and most severely affected sheep (43-06), also had depigmentation of the non-tapetal fundus, which was not seen in the other sheep examined.

The optic disc in sheep is usually ‘kidney shaped’ and heavily myelinated (Maggs, 2008). In three of the affected sheep examined (65-10, 102-09 and 63-08) the appearance of the optic disc was within normal limits. However, in two of the more severely affected sheep (111-08 and 43-06) the optic discs were significantly paler and shrunken when compared to control animals, and had indistinct disc margins.

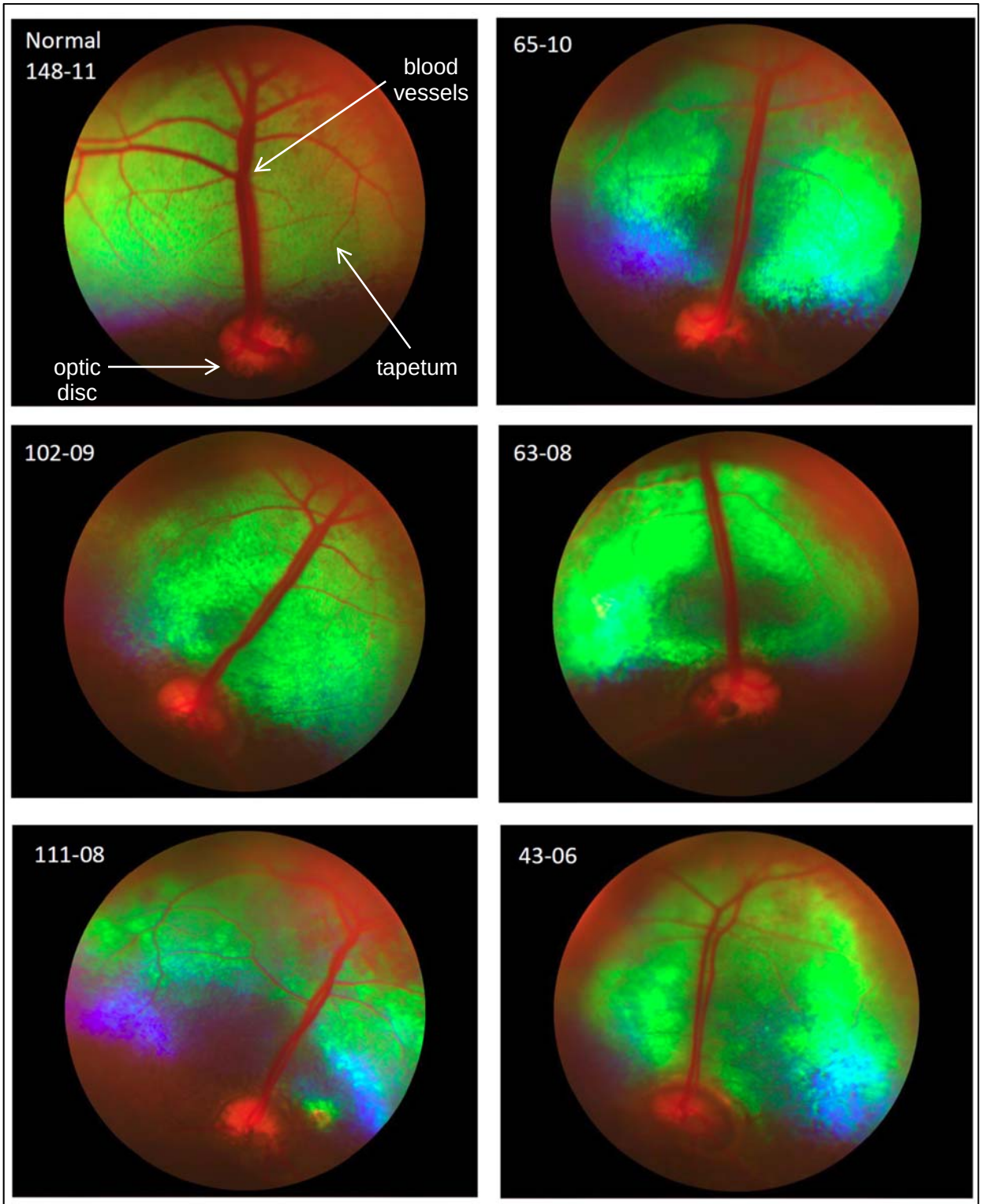

Figure 2.2: **Digital retinal images of affected sheep** captured with the ClearView™ Optical Imaging System (Optibrand, USA). Ordered top to bottom, left to right in order of severity, with a normal sheep in the top left for comparison. There is attenuation of retinal blood vessels and hyperreflectivity of the tapetum in all affected sheep. Sheep 111-08 and 43-06 also exhibit alterations in the size and shape of the optic disc.

2.3.5 HISTOPATHOLOGY

The severity of retinal lesions correlated well with ophthalmologic examination findings in the two cases where histology was performed. In the retina of the 2 year old sheep (65-10), there was complete loss of rod photoreceptors, with no identifiable rod outer segments, inner segements or nuclei seen, as demonstrated in Figure 2.2B. Cone photoreceptors, characterized by their broad, eosinophilic inner segments, were present, but exhibited shortening of the outer segments. The outer nuclear layer was reduced to a single layer of cone nuclei, identifiable based on their mildly vesicular nuclei and position adjacent to the external limiting membrane (Braekevelt, 1983; Samuelson, 2007). Mild attenuation of the outer plexiform layer was observed in this sheep. The inner nuclear, inner plexiform, ganglion cell and nerve fibre layers appeared histologically normal. In the retina of the 6 year old ewe (43-06), similar lesions were observed, but were much more severe in nature, as shown in Figure 2.4B. The photoreceptor and outer nuclear layers of the retina were absent in this sheep, except for occasional small islands of surviving cone photoreceptors. These remaining cone photoreceptors often had no identifiable outer segments, and only very small, blunt remnants of inner segments were present in this sheep. In most fields, the outer plexiform layer was not appreciable, and the retinal pigment epithelium communicated directly with the inner nuclear layer. The inner nuclear, inner plexiform, ganglion cell and nerve fibre layers were not affected. In both sheep, the optic nerve was normal with no evidence of degeneration, and sections of brain examined did not contain significant histological lesions.

Prior to the start of this investigation, eye and brain samples from two other affected ewes (81-05 and 71-07) were collected in Bouins and 10% formalin respectively, and submitted to a commercial diagnostic laboratory (Gribbles Veterinary Pathology, Christchurch) for histological evaluation. The retina in these sheep showed comparable changes to those described in the ewe 43-06, as seen in Figure 2.4A, with only small islands of cone photoreceptors remaining. There were no abnormalities in the sections of brain examined.

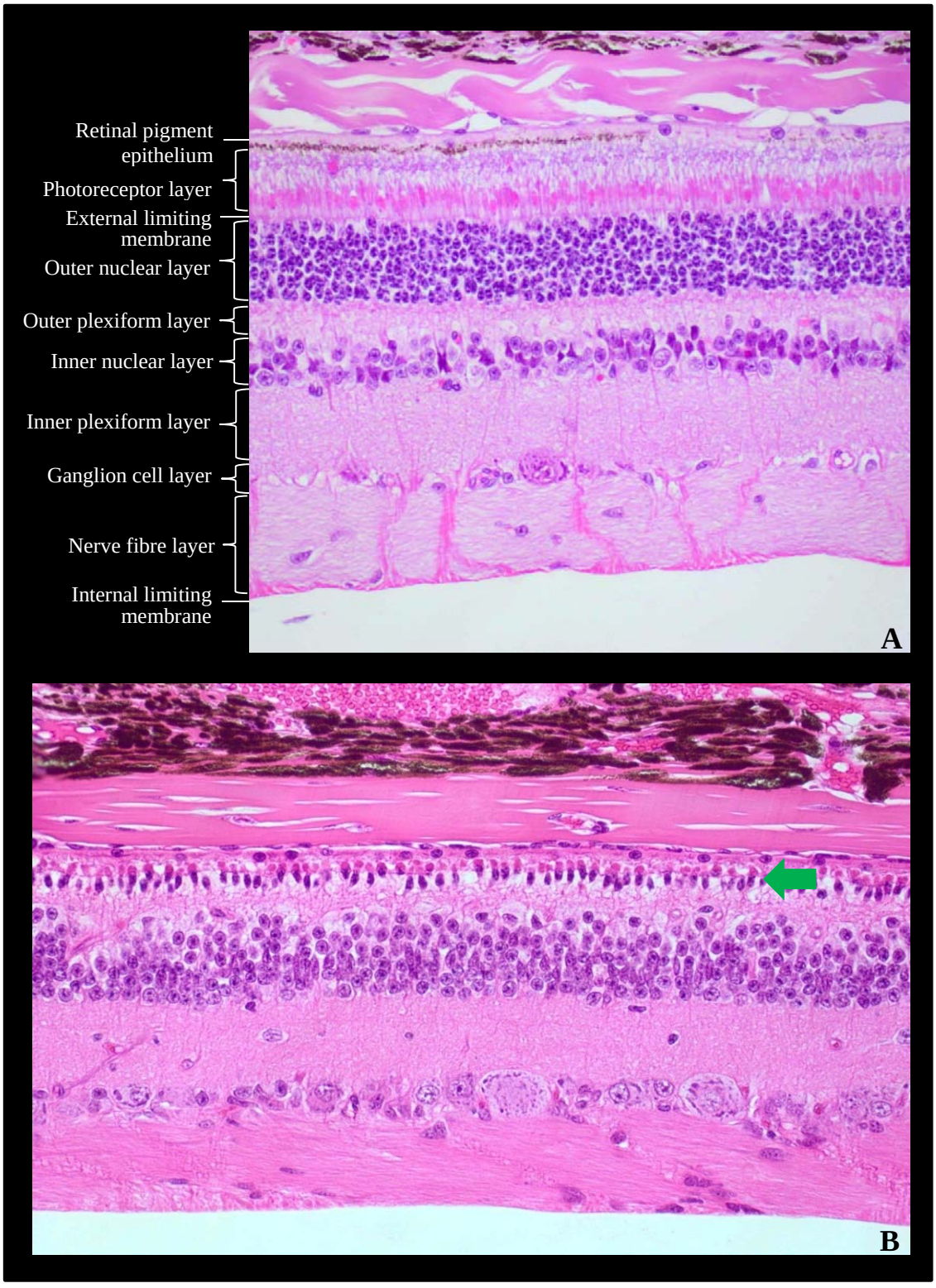

Figure 2.3: **Retinal histology in affected Wiltshire sheep** (H&E, 400x) **A.** Normal sheep retina, with layers labeled; **B.** Retina of a 2-year-old Wiltshire ewe with night blindness. There is severe loss of photoreceptors, with only a single layer of cone photoreceptors remaining (arrow). Other layers of the retina appear normal.

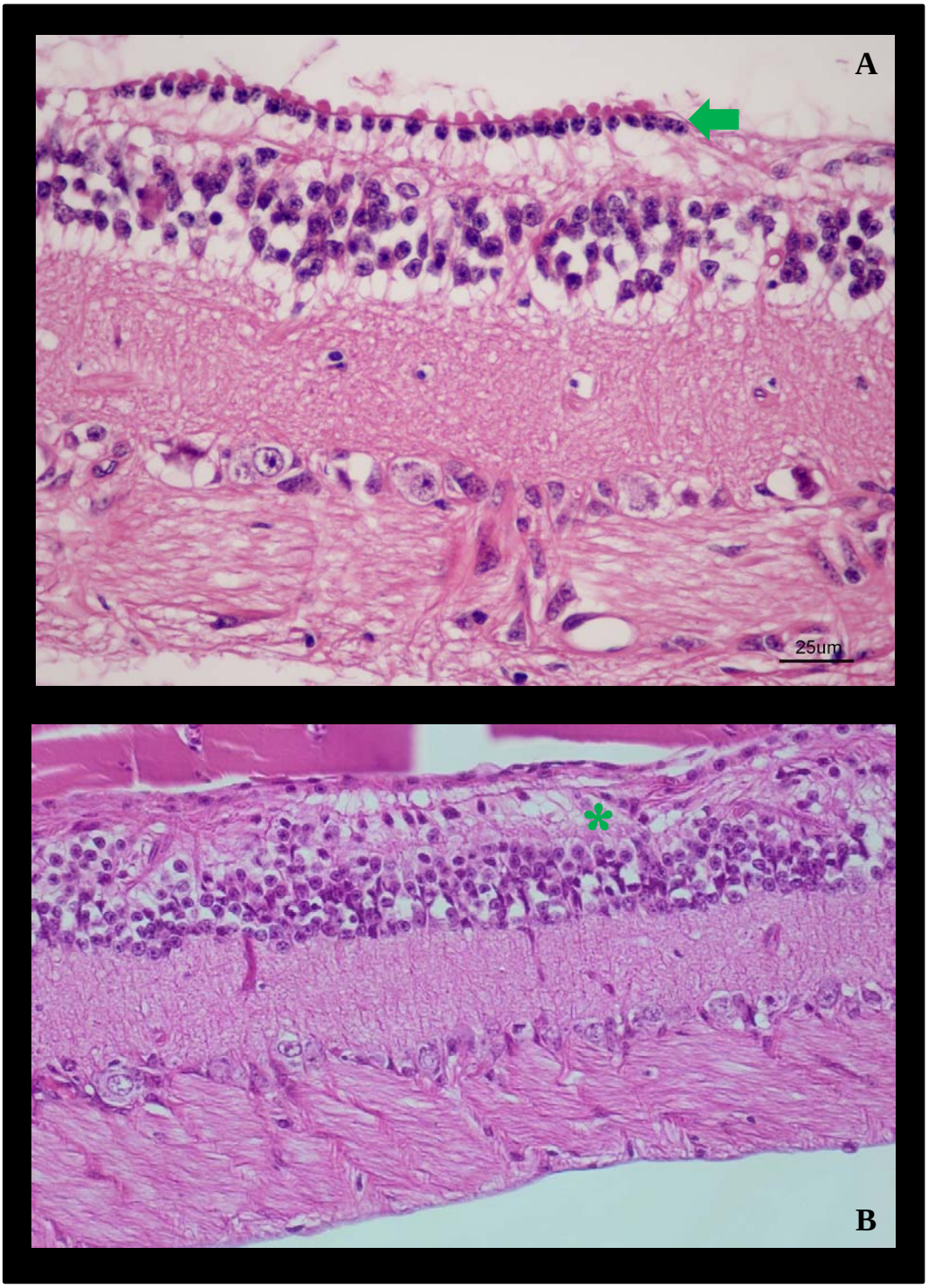

Figure 2.4: **End-stage retinal histology in affected Wiltshire sheep** (H&E, 400x) **A.** Retina from a 6-year-old blind Wiltshire ewe (81-05), showing a small island of surviving cone photoreceptors (arrow). There is artifactual retinal detatchment in this sheep and the retinal pigment epithelium cannot be seen; **B.** Retina of another 6-year-old blind ewe, exhibiting more severe loss of photoreceptors and collapse of the outer plexiform layer (asterix).

2.4 DISCUSSION

The ophthalmologic and microscopic lesions described in these Wiltshire sheep are consistent with a progressive retinal degeneration which primarily targets rod photoreceptors. No lambs or hoggets had any evidence of visual impairment, and the youngest affected ewe was 2 years of age. The degree of visual impairment appeared to increase with age, with the two youngest affected sheep only identified on maze testing in low light conditions, while older affected ewes showed overt signs of blindness when yarded during bright daylight.

Affected sheep showed no overt signs of blindness when grazing, and were in comparable body condition to unaffected sheep. This is in agreement with previous research, which demonstrated that total food intake and productivity were not significantly altered in blinkered sheep that were unable to see what they ate, compared to control sheep (Arnold, 1966). Affected Wiltshire ewes were able to rear lambs successfully, including sets of twins and triplets, even though visual cues have been shown to play a significant role in maternal recognition of offspring in sheep (Alexander, 1977). Ewes also use olfactory and auditory cues to recognize their lambs, but these are usually considered to be of secondary importance to visual cues (Alexander & Shillito, 1977). The flat contour, low stock density and good husbandry on the farm under investigation may have contributed to the high survival rates of lambs born to blind ewes by minimizing opportunities for accidental separation of ewes and their lambs.

As affected ewes did not exhibit any signs of blindness at pasture and were often difficult to identify during mustering, another method was required to detect visually impaired sheep in the flock. The construction of a simple maze within existing yards provided an effective tool for identifying sheep with reduced vision in low light conditions, and performance within the maze correlated well with retinal pathology observed on subsequent ophthalmic examination. Maze testing has been used previously to evaluate the behaviour of Awassi lambs with a novel day blindness (Shamir *et al*., 2010). The design of the maze used in the current study was similar to that described by Shamir *et al*., consisting of a walkway with partial fences, but the maze in the current study had slightly larger dimensions as it was constructed within the existing yards.

For practical reasons and ease of mustering, each mob of 30-40 ewes was subjected to maze testing on different evenings at dusk, and in order to assess sheep under similar ambient light conditions, it was necessary to have three sheep within the maze at any one time. This may have potentially confounded the assessment of visual impairment by allowing affected sheep to closely follow normal sheep, and is a limitation of the current study. However, even when sheep started the maze in close proximity to two other sheep, they were observed to spread out by the time they reached the second obstacle, and any sheep showing hesitation quickly became separated from the others. A further limitation of the current study is that the performance of sheep within the maze was assessed subjectively, rather than recording passage times. In the Awassi lambs with day blindness, passage times through the maze were recorded and affected lambs took significantly longer to complete the maze in light conditions (Shamir, *et al*., 2010), but each lamb had to walk

through the maze three times consecutively and the mean passage time was used for statistical comparisons. This would not have been practical in the current study, as each sheep only completed the maze once and light conditions were not constant. In addition, sheep on this farm (particularly older ewes) were very familiar with people, and so tended to walk through the maze slowly as there was little motivation to move away from people towards the sheep at the other end of the maze.

The fundic examination findings of tapetal hyperreflectivity and attenuation of retinal blood vessels are consistent with retinal degeneration (Ofri, 2008). Thinning of the retina reduces the amount of light absorbed and more light is reflected back to the observer by the tapetum, resulting in tapetal hyperreflectivity. Attenuation of retinal blood vessels is a universal finding in people with retinitis pigmentosa, a group of inherited diseases characterized by degeneration of rod photoreceptors (Hartong, Berson, & Dryja, 2006), but also occurs in other retinopathies. The vasoattenuation seen with photoreceptor degeneration is thought to be the result of alterations in normal oxygen gradients within the retina. Oxygen from the choroid that was once used by the photoreceptors instead diffuses into the inner retina, leading to vasoconstriction, reduced blood flow and reduction in size of the major retinal blood vessels. Capillaries in the inner retina are also permanently lost with photoreceptor degeneration in animals (Penn, Li, & Naash, 2000; Wangsa-Wirawan & Linsenmeier, 2003).

In normal sheep, rod photoreceptors predominate over cones, with 30-40 rods present for every cone (Boileau & Gilmour, 2012). In affected sheep only cone photoreceptors

were observed on retinal histology, as both the outer segments and nuclei of rod photoreceptors had been lost. Rod photoreceptors have a very high sensitivity to light, making them important in scotopic (low light) vision (Bloomfield & Dacheux, 2001) and explaining why affected sheep developed night blindness prior to day blindness. Histologically, the remaining cone photoreceptors in affected sheep exhibited shortened outer segments in the youngest affected sheep, and were lost completely in the oldest affected sheep, a finding that is also reported in primary rod dystrophies in people (Milam, Li, & Fariss, 1998). Changes in the cone photoreceptors are postulated to be due to rod cell death adversely affecting cones via a toxic byproduct, or a loss of trophic factors which are usually produced by rods (Mohand-Said *et al*., 2001), as well as a loss of lateral support.

In other studies investigating retinal disease in sheep, electroretinography has been performed to assess the function of rod and cone photoreceptors (Graydon & Jolly, 1984; Shamir, *et al*., 2010; Smith, Greenlee, Hamir, & Greenlee, 2009). Electroretinography was not performed as part of the current study as sheep need to be anaesthetized for this procedure in order to abolish normal eye movements which alter the baseline recording (Smith III, Witzel, & Pitts, 1976). Facilities on the farm were not suitable for anaesthetizing sheep and electroretinography requires specialized equipment which was not available during the current study, so interpretations of relative rod and cone functionality were based on clinical and histological findings. Any future research into this disease should include electroretinography on affected sheep to confirm the primary involvement of rod photoreceptors, and show how the disease progresses over time based on sequential electroretinograms.

The findings reported here were interpreted to be most consistent with a toxic or inherited retinopathy. Chronic ingestion of bracken fern (*Pteridium spp.*), containing the toxic principle ptaquiloside, can lead to degeneration of the outer retinal layers, including rods, cones and the outer nuclear layer, referred to as 'bright blindness' (Hirono, *et al.*, 1993). However, a thorough search of the property revealed no bracken fern or related plants, and bright blindness has not been previously reported in New Zealand (Smith, 1990). Overdosage with the anthelmintic closantel, a halogenated salicylanilide, can lead to necrosis of rod photoreceptors in sheep, but was ruled out in this case as affected ewes had never been drenched. In addition, no brain lesions were observed in association with the retinal lesions in the cases described here, whereas clostantel toxicity causes status spongiosus of the white matter in the brain and spinal cord, and necrosis, myelin vacuolation and fibrosis of the optic nerve (Van der Lugt & Venter, 2007). The lack of neurological signs and brain lesions also excludes the diagnosis of diseases such as the neuronal ceroid lipofuscinoses (Jolly *et al.*, 1980). Vitamin A deficiency was considered unlikely as a cause of blindness in these sheep, as they are solely pasture-fed and ruminants can efficiently convert beta-carotene from pasture into vitamin A (Commonwealth Scientific and Industrial Research Organisation (CSIRO), 2007). The progression of the retinal lesions with age and the numbers of sheep affected were also not compatible with vitamin A deficiency.

The exclusion of other causes of retinal degeneration in sheep, combined with preliminary pedigree analysis, supported a heritable basis for this disease in Wiltshire sheep. Inherited retinopathies with selective loss of rod photoreceptors have not been

previously described in Wiltshire sheep, or any other sheep breed, but are recognized in other species, particularly humans (Fahim, Daiger, & Weleber, 2000) and dogs (Miyadera, Acland, & Aguirre, 2012). In humans, hereditary retinal diseases with primary degeneration of rod photoreceptors are well described and are referred to as retinitis pigmentosa (Voaden, 1991). The clinical course of the disease in sheep closely parallels what has been described with retinitis pigmentosa in humans, and both diseases have similar ophthalmic examination and histological findings (Milam, *et al.*, 1998; Hartong, *et al.*, 2006). Most commonly, people with retinitis pigmentosa (RP) experience the onset of night blindness during adolescence, with loss of the mid-peripheral visual field. Over time, far peripheral vision is lost leading to tunnel vision, and most patients are considered legally blind by 40 years of age, with complete blindness by 60 years (Hartong, *et al*., 2006; Milam, Li, & Fariss, 1998). Shorting of the outer segment of rod photoreceptors is the initial histological lesion observed in all forms of RP, and as rod photoreceptors degenerate and die there are reduced numbers of nuclei in the outer nuclear layer, as observed in affected sheep (Milam, *et al*., 1998). Cone degeneration occurs following the loss of neighbouring rods (Mohand-Said *et al*., 2001), and loss of photoreceptors is accompanied by hypertrophy of Müller cells and migration of cells from the retinal pigment epithelium into the inner retina, reducing the area of the subretinal space (Milam, *et al*., 1998). Retinitis pigmentosa is a genetically heterogeneous group of diseases and to date over 65 genes have been implicated, with autosomal dominant, autosomal recessive, X-linked and non-Mendelian forms of inheritance (Anasagasti *et al.*, 2012). The possible genetic basis for the retinal degeneration in Wiltshire sheep will be discussed further in Chapter 3.

2.4.1 SUMMARY

The history, ophthalmic examination and histological findings described in Wiltshire sheep in this study are consistent with a progressive retinal degeneration targeting rod photoreceptors. Affected sheep have normal vision at birth, but develop night blindness at 2 to 3 years of age due to selective loss of rod photoreceptors. Over time, this progresses to complete blindness due to secondary loss of cone photoreceptors, and advanced tapetal and retinal vascular changes are observed on fundic examination. The information available supports an inherited basis for this disease, and there are numerous similarities between this disease, retinitis pigmentosa in humans and progressive retinal atrophy in dogs. The findings reported in this chapter confirm that the blindness in Wiltshire sheep represents a novel retinopathy not previously described in any breed of sheep.

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Chapter 3

INHERITANCE & MOLECULAR GENETICS OF BLINDNESS IN WILTSHIRE SHEEP

3.1 INTRODUCTION

Inherited retinal diseases have been rarely described in sheep, and are limited to day blindness in Awassi sheep (Shamir *et al*., 2010), congenital microphthalmia in Texel lambs (De Groot, 1957; Hanset, 1961) and ceroid lipofuscinosis in New Zealand Borderdale and South Hampshire sheep (Jolly *et al.*, 1980). Of these, the only disease which affects the retina in isolation is day blindness in Awassi sheep, a form of achromatopsia characterised by cone dysfunction rather than loss. Microphthalmia is a dysplasia affecting all embryonic components of the eye (Roe *et al.*, 2003), while ceroid lipofuscinosis results in the accumulation of storage products in numerous tissues (Jolly, *et al*., 1980).

Clinical findings in Wiltshire sheep with adult-onset blindness were consistent with an inherited retinal degeneration targeting rod photoreceptors. Inherited

retinopathies characterised by selective loss of rod photoreceptors have not been previously reported in sheep, but are well recognised in people, where they are collectively known as retinitis pimentosa (Hamel, 2006), and dogs, in which progressive retinal atrophy (PRA) is reported in over 100 breeds (Miyadera, Acland, & Aguirre, 2012). Worldwide, retinitis pigmentosa (RP) affects approximately 1 in 4000 people, and to date, over 65 different genes have been implicated (Hartong, Berson, & Dryja, 2006), However, these account for only 60% of RP cases, and the mutations involved in the remaining 40% of cases are currently unknown (Anasagasti *et al.*, 2012). In addition, different mutations in the same gene may cause different diseases and forms of retinitis pigmentosa, adding to the genetic complexity (Ferrari *et al*., 2011). Inheritance of retinitis pigmentosa can be autosomal dominant (accounting for approximately 30-40% of cases), autosomal recessive (50-60% of cases) or X-linked (5-15%), although rarer forms with non-Mendelian inheritance also exist (Hartong, *et al*., 2006). The majority cases are confined to the eye and are referred to as simple, or non-syndromic RP, in order to distinguish them from syndromic RP, in which other organs are also affected, and systemic RP, where the retinal disease is secondary to systemic pathology (Ferrari, *et al*., 2011). Syndromic and systemic RP together represent approximately 25% of all retinitis pigmentosa cases (Daiger, Bowne, & Sullivan, 2007). The most common syndromic forms of RP are Usher syndrome, where retinal lesions are accompanied by sensorineural hearing loss, and Bardet-Biedl syndrome, characterised by retinitis pigmentosa with obesity, polydactyly, hypogonadism, mental retardation and renal failure (Anasagasti, *et al*., 2012).

Most of the genes implicated in retinitis pigmentosa are responsible for only a small proportion of cases, with rhodopsin a notable exception to this. Mutations in

rhodopsin (*RHO*) can cause both autosomal dominant and autosomal recessive retinitis pigmentosa, and in a study of predominantly American and European families, *RHO* mutations accounted for over 25% of all cases of autosomal dominant RP (Sullivan *et al.*, 2006; Daiger, *et al.*, 2007). The same study revealed that the *RDS* (peripherin) gene is another common locus for mutations in autosomal dominant RP, with 9.5% of cases attributable to changes in this gene. *RDS* is important in the development and maintenance of photoreceptor outer segments; mice homozygous for a mutation in *RDS* develop no outer segments, while heterozygotes have shortened, disorganised outer segments (Van Soest *et al.*, 1999).

There are 26 genes currently associated with autosomal recessive retinitis pigmentosa and of these, mutations in the gene *USH2A,* coding for the basement membrane protein Usherin, appear to have the highest prevalence. Usherin mutations account for 10-15% of all autosomal recessive RP cases, as well as 30-40% of Usher type II cases, a syndromic form of RP. Other important genes in autosomal recessive retinitis pigmentosa (arRP) include *CRB1,* encoding Crumbs homolog 1, a transmembrane protein which is associated with 6.5% of arRP cases; *PDE6A* and *PDE6B,* which account for approximately 4% of arRP cases each, and encode for cyclic GMP phosphodiesterase subunits that are vital in the process of phototransduction; and *RPE65,* responsible for a microsomal protein in the retinal pigment epithelium and associated with 2% of cases (Daiger, *et al*., 2007; Ferreyra & Heckenlively, 2012). In X-linked retinitis pigmentosa, mutations in the *RPGR* gene, coding for the RP GTPase regulator protein, are found in 70-75% of patients with this disease (Hartong, *et al.*, 2006; Daiger, *et al.*, 2007).

The aim of the present study was to investigate the genetic basis underlying the retinal degeneration in Wiltshire sheep, using a candidate gene approach of sequencing the rhodopsin gene in affected sheep, as well as homozygosity mapping to identify chromosomal regions identical by descent.

3.2 MATERIALS AND METHODS

3.2.1 RHODOPSIN

3.2.1.1 ANIMALS USED AND SAMPLE COLLECTION

The subjects were three female Wiltshire sheep, aged 4 to 6 years, with retinal degeneration confirmed on ophthalmic examination. A 6 year old ophthalmologically normal female Wiltshire sheep from the same flock, and an unrelated Perendale sheep from a different property, were used as control subjects. Blood was collected via jugular venipuncture into an evacuated tube containing lithium heparin (BD Vacutainer ® Glass tube 10 mL, Becton, Dickinson and Company, New Jersey, USA).

3.2.1.2 PRIMER DEVELOPMENT

DNA polymerase chain reaction (PCR) primers for sheep rhodopsin (*RHO*) were designed based on the cattle *RHO* mRNA sequence (NCBI Reference Sequence: NM_001014890.1). This sequence was analysed against the Ovine (Texel) version 2.0 Genome Assembly (Livestock Genomics) using the 'BLAST' function, and matches were found on chromosome 19 (OAR19), corresponding to the five exons of *RHO.* These sequences, and approximately 500 adjacent nucleotides, were extracted in fasta format, and the reverse complement sequences generated using Geneious Pro 5.5.7 (Biomatters). Primers were then created for each of the exons using the NCBI Primer-BLAST tool. Exons 3 and 4 were able to be amplified by a single primer, as these are

separated by an intron that is only 118 base pairs long. Details of the primers created are listed in Table 3.1. Subsequent to this experiment, the predicted sheep *RHO* mRNA sequence was published (NCBI Reference Sequence XM_004018534.1) as part of the Ovis Aries Annotation Release 100, and there were no significant differences between this sequence and that used in the generation of primers.

Table 3.1: **List of primers used for PCR and sequencing of *RHO***

Primer Name	**Sequence (5'-3')**	**T_m**	**Product size**
RHO exon 1 forward	CCCCACCTGGAAGCCAATTA	59.82	677
RHO exon 1 reverse	TGTCCACCATCTGCAAGGTC	59.96	
RHO exon 2 forward	GCGAGCTGATTGCCATGTTC	60.25	608
RHO exon 2 reverse	CCCGTAGTGACGTTAGGAGC	59.90	
RHO exon 3 & 4 forward	CGCTGAACAAGGCAACAACA	59.90	893
RHO exon 3 & 4 reverse	ACTCATGATCACCCCAGGGA	59.96	
RHO exon 5 forward	GTTTGGTCCCAGCCATCTGA	59.96	648
RHO exon 5 reverse	TCTCTCCATCCACGTCTCCA	59.67	

RHO = rhodopsin

3.2.1.3 DNA EXTRACTION

DNA was extracted from blood using a DNeasy® Blood and Tissue Kit as per the manufacturer's instructions outlined in the Spin-Column Protocol (Qiagen N.V., Netherlands). DNA concentration was measured with both a Qubit 2.0 Fluorometer (Life Technologies Corporation, USA) and a NanoDrop 2000 Spectrophotometer at a wavelength of 260 nm (Thermo Fisher Scientific, USA). Extracted DNA was stored at -18°C until required.

3.2.1.4 POLYMERASE CHAIN REACTION

The extracted DNA (2.5 µL) was used to amplify the *RHO* gene as follows: the 25 µL PCR reaction mix contained 1 x PCR buffer, 2 mM $MgCl_2$, 0.2mM each dNTP, 0.2 mM each primer and 0.5 units of Taq-Ti DNA polymerase (Fisher Biotec, Australia). A separate reaction mix for each sheep was set up for each of the four primer pairs outlined in section 3.2.2. DNA extracted from the normal Wiltshire sheep and unrelated Perendale sheep were used as positive controls. In all reactions, negative controls were performed to check for the presence of contaminants. The PCR conditions used are outlined in Table 2, with reactions taking place in a Veriti 96 Well Thermal Cycler (Applied Biosystems, USA).

Table 3.2: **PCR conditions for sequencing of *RHO***

Exon	PCR conditions
Exon 1	95°C for 10 min; 40 cycles of 30s at 95°C, 30s at 60°C and 1 min at 72°C; 72° for 5 min
Exon 2	95°C for 10 min; 35 cycles of 30s at 95°C, 30s at 58°C and 1 min at 72°C; 72° for 5 min
Exon 3/4	95°C for 10 min; 40 cycles of 30s at 95°C, 30s at 60°C and 1 min at 72°C; 72° for 5 min
Exon 5	95°C for 10 min; 40 cycles of 30s at 95°C, 30s at 63°C and 1 min at 72°C; 72° for 5 min

All PCR products were analysed on a 1% (w/v) ultra-pure agarose gel (Axygen Biosciences, USA) containing ethidium bromide and visualised under UV light on a E-Gel® Imager transilluminator (Life Technologies Corporation, USA).

3.2.1.5 SEQUENCING REACTIONS

Amplicons of exons 1, 2 and 5 to be used for sequencing were purified using the PureLink™ PCR Purification Kit (Invitrogen Corporation, USA). Amplicons of exon 3 and 4 were purified using the PureLink™ Quick Gel Extraction Kit (Invitrogen

Corportion, USA) according to the manufacturer's instructions. Purified amplicons were combined with the sequencing primers in Table 1 and subjected to automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 DNA Analyzer (Applied Biosystems Inc., USA).

3.2.2 HOMOZYGOSITY MAPPING

A genome wide association study was conducted using the Illumina OvineSNP50 BeadChip (Illumina, San Diego, CA, USA) on the DNA of 6 Wiltshire sheep, including 5 sheep with confirmed retinal degeneration on ophthalmic examination and one opthalmologically normal Wiltshire sheep. Standard procedures were followed with a PCR and ligation free protocol. The OvineSNP50 BeadChip analysis was performed in association with Dr. Dorian Garrick (Iowa State University, Iowa, USA) to define homozygous regions with consecutive single-nucleotide polymorphism (SNP) loci only existing in all the affected sheep.

3.3 RESULTS

3.3.1 PEDRIGREE ANALYSIS

The breeder maintains comprehensive mating and lambing records, which were used to construct pedigree charts for the nine ewes identified with retinal degeneration during 2011 and 2012 (Appendix, Figure A.1). No pedigree information was available for rams that were bought onto the property. The Ardo and HH rams appear in the pedigrees of all affected sheep, but these also feature in the pedigrees of most of the normal-sighted sheep on the property, so the significance of this is uncertain. Neither of these rams showed signs of visual impairment whilst on the property, but HH was only

kept for a short period and used in the 2005 breeding season, when he was 2 years of age. The youngest animal to develop retinal degeneration (65-10) was the daughter of a ewe which also had confirmed retinal degeneration, but none of the other affected ewes had direct ancestors known to be affected. Depending on what assumptions are made when analysing the pedigrees, either an autosomal dominant, autosomal recessive or complex form of inheritance would be possible.

The small size of the flock and limited numbers of affected animals identified meant it was not possible to conduct a segregation analysis to test whether or not the occurrence of the disease was compatible with a simple Mendelian form of inheritance (Nicholas, 1987). The Singles Method of segregation analysis uses phenotypic data, including the number of affected offspring, the total number of offspring, and the number of families with one or two affected offspring, to estimate the segregation frequency and its variance. The segregation frequency of the disease can then be tested against the expected segregation frequency for a particular mode of inheritance (the null hypothesis), which is based on Mendelian principles. In the Wiltshire sheep, the total number of offspring is known, but the number of affected offspring is likely to be grossly underestimated, as a high proportion of sheep born on the property are sold or culled at a young age, before retinal degeneration can develop at 2-3 years of age. Additionally, the breeder recognises that cases of retinal degeneration may have been overlooked in the past, as visual impairment is not readily noticeable in sheep in a pastoral farming situation, and several affected animals were only detected during maze testing.

3.3.2 RHODOPSIN

The amplicons obtained from PCR using the *RHO* exon 1 primers were larger than predicted. Sequencing showed that an additional 28 bases were present in the intron, prior to the start of exon 1, in both affected and control sheep compared to the reference sheep (*Ovis aries*) genome (Figure 3.1). The extra 28 bases were inserted between 56,092,161 and 56,092,162 on chromosome 19 (OAR_v3.1), and exon 1 begins 55 base pairs along from this at position 56,092,106. There were also 5 single nucleotide polymorphisms (SNPs) in the intronic region between the inserted bases and the start of exon 1 when the reference sheep genome was compared to the sequence obtained in affected and control sheep. Amplicons from PCR of other exons of *RHO* were of the lengths predicted in Table 3.1.

A single nucleotide polymorphism (SNP) was present in both affected and control sheep in exon 1 of *RHO*, compared to the reference sheep genome in OAR_v3.1. At position 56,091,843 on chromosome 19, there is a thymine (T) in the reference genome, while sequencing of exon 1 in the affected and control sheep showed a cytosine (C) in this position. This does not impart any change to the resulting amino acid sequence as the altered base is the third base of a codon, and both UUU and UUC encode phenylalanine.

A further SNP was present in exon 5 of *RHO*, at position 56,087,343 on chromosome 19. The reference sheep genome indicates there is a cytosine (C) in this position, but control and affected sheep had a thymine (T) instead. This SNP is also the third base of a codon and does not change the resulting amino acid sequence, as both

GAC and GAU code for aspartic acid. Amplicons from PCR of *RHO* exons 2, 3 and 4 in control and affected sheep did not differ from the reference sheep genome sequence.

3.3.3 HOMOZYGOSITY MAPPING

All markers that were monomorphic in the affected and normal sheep were excluded from the analysis, leaving 1907 markers that were monomorphic in the five affected Wiltshire sheep. The largest homozygous segment consisted of 45 consecutive SNP loci, starting from SNP OAR5_96674157.1 and extending to SNP s59216, covering a region of 4.85 Mbp (OAR_ v3.1: 88,634,982 to 93,480,111) on the long arm of chromosome 5. There were 29 genes located in this region based on the ovine reference sequence within the NCBI Map Viewer (OAR_v3.1, annotation release 100). In order to include any genes located in the immediate vicinity of the homozygous SNP segment, the search area was extended from 88,000,000 to 94,000,000, and an additional 8 genes were identified (Table 3.3). None of the genes within 45-SNP region were considered plausible candidate genes based on their biological functions. However, a gene in the extended search area, G-protein coupled receptor 98 (*GPR98*) may warrant further investigation as it has been implicated in Usher syndrome type 2 in people, which is characterised by retinitis pigmentosa and sensorineural hearing loss. The *GPR98* gene in the sheep is located on chromosome 5, and spans over 523 kbp from 87499346 to 88022860. The coding sequence is 18,957 base pairs long and in humans, the gene contains over 90 exons, making it one of the largest in the entire genome.

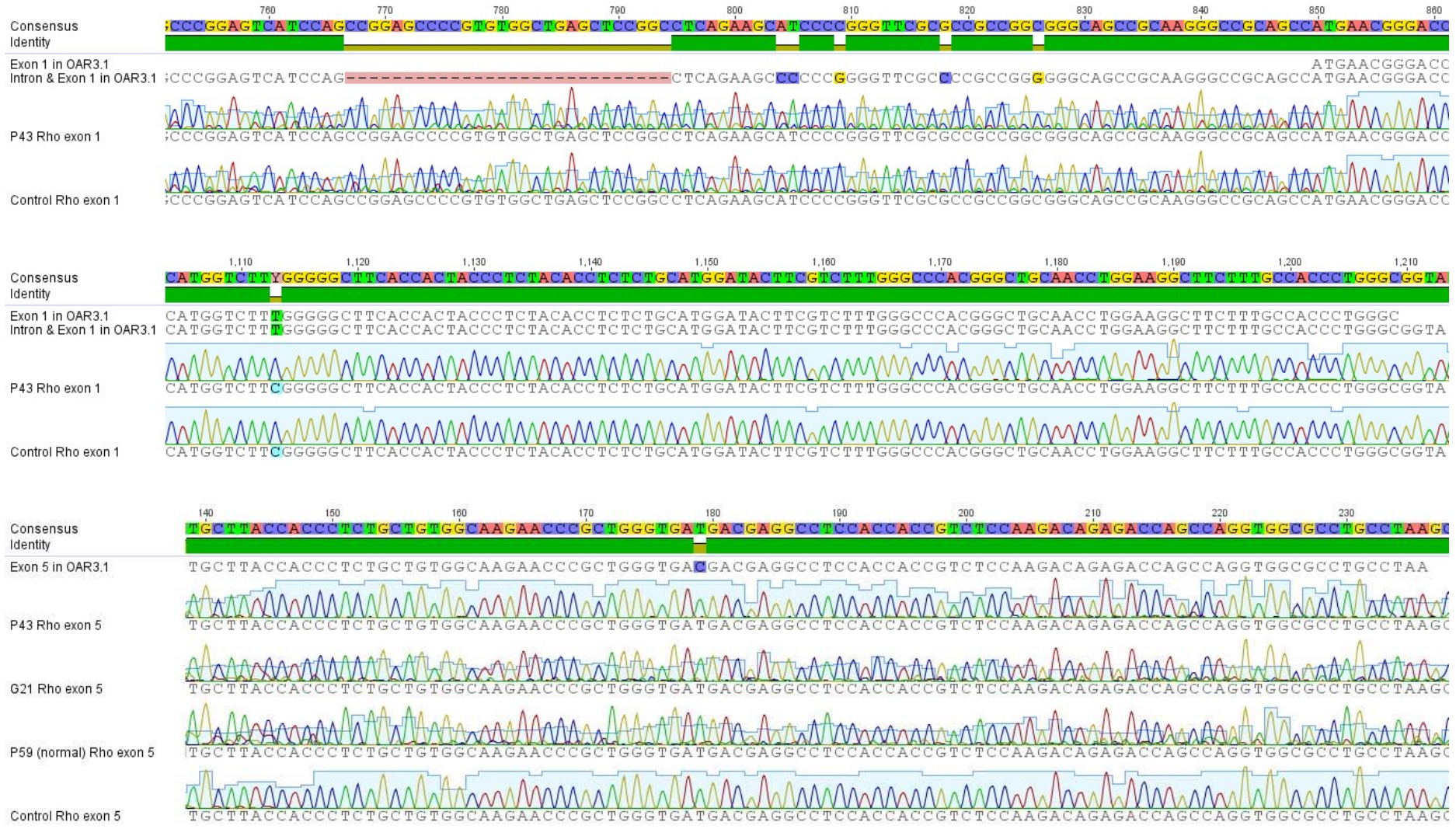

Figure 3.1: **Sequence of selected regions of the rhodopsin gene in affected and normal sheep** from Geneious version 7.0, Biomatters. **A.** Intron preceding exon 1, showing insertion of 28 bases (dashes) and five SNPs (yellow and purple highlights) in blind (P43) and control sheep, relative to the reference sheep genome (OAR 3.1); **B.** SNP (C vs. T, blue highlight) in exon 1 of blind (P43) and control sheep relative to reference sheep genome; **C.** SNP (T vs. C, purple highlight) in exon 5 of blind sheep (P43, G21) and normal sheep (P59, control) relative to the reference sheep genome. SNP = single nucleotide polymorphism

Table 3.3: **Genes within and adjacent to the largest homozygous segment, on homozygosity mapping of five affected sheep using the Illumina OvineSNP50 BeadChip**
(SNP OAR5_96674157.1 to SNP s59216 on chromosome 5)

Ovine Chromosome Position (OAR v3.1)	Ovine Reference Gene Symbol	Ovine Reference Gene Name
OAR5: 87499346..88022860	GPR98	G protein-coupled receptor 98
OAR5: 88196161..88210276	**ARRDC3**	arrestin domain containing 3
OAR5: 88812082..88812503	**LOC101107352**	ribosomal protein L23 pseudogene
OAR5: 89941245..89943167	**LOC101121010**	envelope glycoprotein-like
OAR5: 90189070..90193317	**LOC101121262**	tyrosine-protein kinase ABL1-like
OAR5: 90395656..90407332	**NR2F1**	nuclear receptor subfamily 2, group F, member 1
OAR5: 90474547..90475270	**LOC101107597**	60S ribosomal protein L10a-like
OAR5: 90539456..90540896	**POU5F2**	POU domain class 5, transcription factor 2
OAR5: 90561557..90837228	**FAM172A**	family with sequence similarity 172, member A
OAR5: 90949863..90950362	**LOC101108129**	ribosomal protein S25 pseudogene
OAR5: 91234503..91234575	**TRNAC-ACA**	transfer RNA cysteine (anticodon ACA)
OAR5: 91210141..91294157	**KIAA0825**	KIAA0825 ortholog
OAR5: 91294161..91367053	**ANKRD32**	ankyrin repeat domain 32
OAR5: 91377594..91683461	**MCTP1**	multiple C2 domains, transmembrane 1
OAR5: 92018189..92018743	**LOC101122786**	translocase of inner mitochondrial membrane 17 homolog A (yeast) pseudogene
OAR5: 92025655..92025726	**TRNAR-CCU**	transfer RNA arginine (anticodon CCU)
OAR5: 92035436..92089062	**FAM81B**	family with sequence similarity 81, member B
OAR5: 92108031..92209512	**TTC37**	tetratricopeptide repeat domain 37
OAR5: 92209775..92265560	**ARSK**	arylsulfatase family, member K
OAR5: 92307803..92307874	**TRNAC-ACA**	transfer RNA cysteine (anticodon GCA)
OAR5: 92319218..92321792	**RFESD**	Rieske (Fe-S) domain containing
OAR5: 92323904..92351106	**SPATA9**	spermatogenesis associated 9
OAR5: 92351918..92354571	**LOC101123299**	up-regulated during skeletal muscle growth protein 5-like
OAR5: 92361483..92441147	**RHOBTB3**	Rho-related BTB domain containing 3
OAR5: 92467076..92469946	**LOC101109877**	uncharacterized LOC101109877
OAR5: 92549670..92622356	**ELL2**	elongation factor, RNA polymerase II, 2
OAR5: 92913631..92913702	**TRNAC-ACA**	transfer RNA cysteine (anticodon ACA)
OAR5: 93020527..93047970	**LOC101102236**	ethanolaminephosphotransferase 1-like
OAR5: 93068420..93117383	**PC1**	prohormone convertase 1 precursor
OAR5: 93334684..93334755	**TRNAW-CCA**	transfer RNA tryptophan (anticodon CCA)
OAR5: 93394472..93483934	**CAST**	calpastatin
OAR5: 93487472..93524227	ERAP1	endoplasmic reticulum aminopeptidase 1
OAR5: 93627089..93671124	ERAP2	endoplasmic reticulum aminopeptidase 2
OAR5: 93730682..93785091	LNPEP	leucyl/cystinyl aminopeptidase
OAR5: 93804381..93805241	LOC101110759	FUN14 domain-containing protein 1-like
OAR5: 93857787..93911227	LIX1	Lix1 homolog (chicken)
OAR5: 93921905..93942422	RIOK2	RIO kinase 2 (yeast)

Genes within the homozygous region are in bold. GPR98 is highlighted in red as this has been implicated in Usher syndrome type 2 in people

3.4 DISCUSSION

All Wiltshire sheep diagnosed with progressive retinal degeneration were purebred polled Wiltshire ewes, which were born on the same property between 2006 and 2010. Wiltshire sheep were first introduced to New Zealand in 1972, when four polled ewes and a horned ram were imported from Australia (Morrison Farming). The breed is characterised by an ability to naturally shed its fleece, so numbers of ewes tend to increase when the price of wool is low and lamb is highly valued, as Wiltshire sheep generally produce fast-growing, well-muscled lambs. In 1984, all the purebred Wiltshire females in the country at that time, totalling 50 ewes and 40 ewe lambs, were assimilated into one flock by the Morrison family of Marton. Three Wiltshire horn ewes and one ram were imported into this flock from Australia, and since then very low numbers of Wiltshire sheep have been imported. As a consequence, the genetic pool of pure Wiltshire sheep in New Zealand is narrow, although there are numerous Wiltshire-cross sheep, as Wiltshire rams are used across other breeds in some commercial operations. With the exception of several cases of cerebellar cortical abiotrophy in lambs on a hobby farm during 2000-2002 (Johnstone *et al.*, 2005), no inherited diseases have been reported to occur primarily in the New Zealand Wiltshire population.

The rod-specific retinal degeneration identified in Wiltshire sheep in this study is thought to be inherited, as it occurs in related sheep and there is a predictable, age-related progression of disease. Additionally, all known toxic, nutritional and infectious causes of retinal degeneration were excluded in affected sheep on the basis of the clinical history, ophthalmic examination, and microscopic findings. In order to investigate the molecular genetics underlying this disease, a comparative candidate gene approach was initially used. This strategy relies upon knowledge of similar diseases or

phenotypic traits in other species, which are caused or influenced by functionally conserved or structurally homologous genes (Zhu & Zhao, 2007). The retinal degeneration in Wiltshire sheep had significant similarities to retinitis pigmentosa in humans and progressive retinal atrophy in dogs, and the organisation and function of the retina is largely conserved between vertebrate species (Dowling, 1987). Therefore, the investigation into the disease in Wiltshire sheep was suited to a comparative candidate gene approach. Furthermore, homozygosity mapping and other genetic variant detection methods could not be used at the start of this investigation as there were initially only three affected animals from which DNA could be obtained, which is insufficient to obtain meaningful results from these methods.

Rhodopsin was chosen as a candidate gene in this study due to the central role it plays in rod phototransduction across vertebrate species (Figure 3.2), and its involvement in primary rod photoreceptor degenerations in other species. The process of phototransduction begins with absorption of light by the 11-*cis* retinal chromophore of rhodopsin, which is isomerised to all-*trans* retinal (Pepe, 1999). Photoexcited rhodopsin then activates the rod G protein, transducin (G_t), and catalyzes the exchange of GTP for GDP. The resulting GTP-activated G complexes activate phosphodiesterase (PDE), which in turn hydrolyzes cGMP, reducing its cytoplasmic concentration. This leads to the closure of cGMP-gated Na^+ channels in the plasma membrane and hyperpolarisation of photoreceptors, generating signals transmitted to downstream neurons (Pepe, 1999; Shichi, 2006; Miyadera*, et al.*, 2012). Therefore, rhodopsin, transducin and phosophodiesterase (PDE6A & PDE6B), along with the rod cGMP-gated cation channels, are vital in rod phototransduction and mutations in the genes encoding these proteins can result in degeneration of rods. In particular, mutations in

rhodopsin are associated with a significant proportion (approximately 25%) of cases of autosomal dominant retinitis pigmentosa in humans, as well as 1% of autosomal recessive retinitis pigmentosa cases (Hartong, *et al*., 2006). As the retinal degeneration in Wiltshire sheep shares a number of similarities with retinitis pigmentosa, rhodopsin was considered a strong candidate gene to sequence in affected sheep.

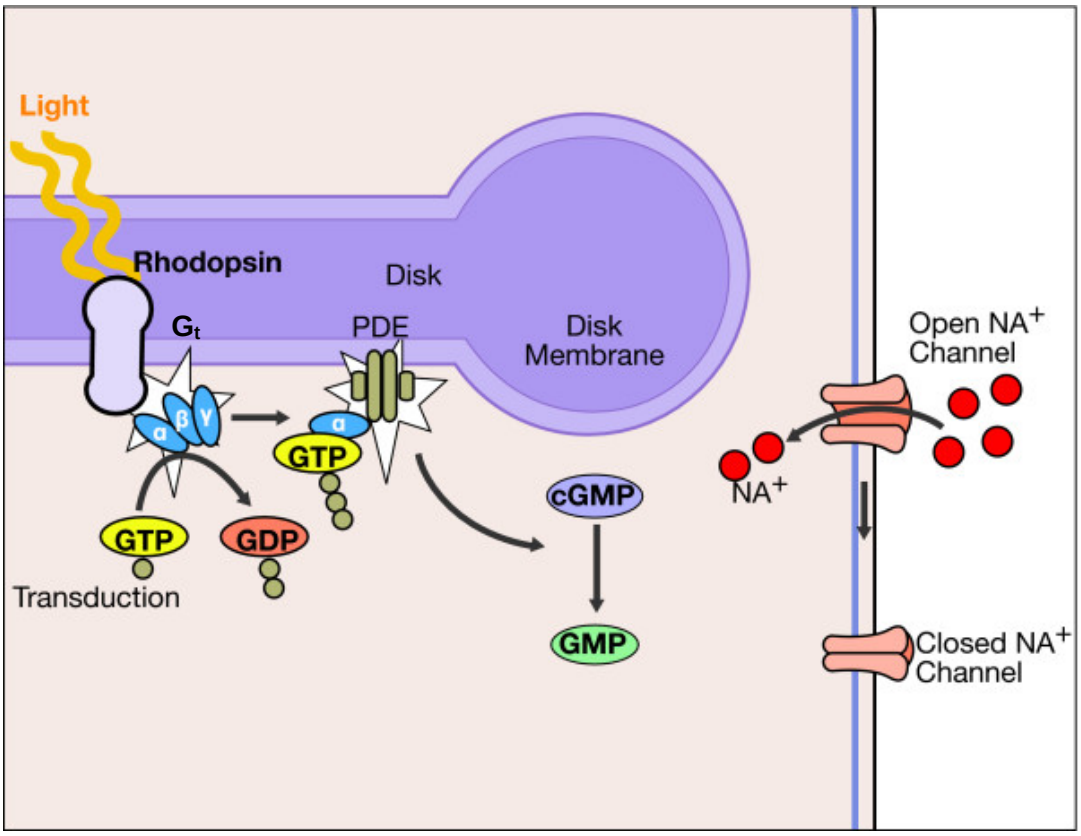

Figure 3.2: **Schematic diagram of phototransduction in rod photorecptors.** G_t = transducin, PDE = phosphodiesterase. Adapted from Konieczka, Flammer, Todorova, Meyer, & Flammer (2012)

However, in the present study, amplification and sequencing of the rhodopsin gene in affected Wiltshire sheep failed to reveal any disease-associated mutations within coding regions. When compared with the reference sheep genome in OAR_v3.1, all sheep sequenced had 28 additional base pairs in the intron preceding exon 1, as well as five single nucleotide polymorphisms in the region between these additional base pairs

and the start of exon 1. Mutations within the introns of the rhodopsin gene, particularly at mRNA splice sites, have been associated with retinitis pigmentosa in humans (Macke *et al.*, 1993; Bell *et al.*, 1994; Jacobson *et al.*, 1994; Rosenfeld *et al.*, 1995; Reig *et al.*, 1996; Greenberg, Roberts, & Ramesar, 2003). However, the sequence differences between the reference sheep genome and sheep included in this study are not considered to be of any clinical significance, as control sheep, including a 6 year old Wiltshire ewe with normal vision and an unrelated Perendale sheep, had identical rhodopsin sequences to the blind Wiltshire ewes. The two single nucleotide polymorphisms found within exon 1 and exon 5 of the rhodopsin gene were also present in both control and affected sheep, and did not impart any changes to the resulting amino acid sequence.

The possibilities of an intronic mutation which alters the function of rhodopsin, or a mutation causing a quantative change in the level of rhodopsin synthesis, cannot be excluded based on the results of this study. To address this, future research directions could include sequencing of the intronic regions of the rhodopsin gene, and quantification of rhodopsin mRNA expression in the retina of affected sheep.

One of the main limitations in investigating the underlying cause of the disease is the low number of affected sheep identified, and the late age of onset of clinical signs. It is likely that the sheep diagnosed with progressive retinal degeneration as part of this research represent only a small proportion of the total number of affected sheep over recent years. Many of the sheep born on the property, particularly ram lambs, are sold or sent to slaughter at a young age, well before retinal degeneration would be expected to develop. Additionally, cases may have been overlooked in sheep which remained on the farm, as observations of affected sheep have shown they are adept at compensating for

their vision loss in a flock situation. Blindness only becomes apparent when affected sheep are on their own in low light conditions, which is not a common occurrence in an extensive farming system. For these same reasons, retinal degeneration may be present undetected in other Wiltshire flocks, especially those where sheep are grazed in large mobs. Underreporting may also be a significant issue, as in general farmers are unlikely to seek veterinary assistance for a single suspected blind animal, and it is only when multiple cases occur that the problem is investigated.

The low number of sheep identified with progressive retinal degeneration made it difficult to determine the probable mode of inheritance. If the disease was inherited in an autosomal dominant manner, every affected animal should have an affected parent (Nicholas, 1987), which was not observed in this flock, as shown in the pedigree charts included in Appendix Table A.1. However, as outlined in the previous paragraph, the number of affected sheep is unlikely to be limited to those identified in this research, and a dam or sire of a known blind sheep may have been affected, but not identified as such. Two main rams feature in the pedigrees of most sheep on the farm, including all the sheep with confirmed retinal degeneration. The main foundation ram of the flock is Ardo, purchased from one of the largest Wiltshire studs in New Zealand. If he was the primary source of a dominant mutation causing retinal degeneration, most of the flock would be expected to be blind and this is not the case. A recessive inheritance pattern involving Ardo is possible, but requires the assumption that other rams used in subsequent years (HH and AV) were carrying the same mutation. The other ram that features in all the pedigrees of affected sheep is HH, who was used for mating in 2005 in an effort to increase muscling. He was purchased from a breeder who buys stock from a number of sources and does not keep detailed pedigree records, so it is not

known whether he shared any parentage with the Ardo ram. One of the lines of rams descended from HH, involving his son (187-05) and grandson (163-06), feature in the pedigrees of five out of nine known blind sheep, and it is plausible that a dominant mutation could have been passed down this genetic line. None of these rams were identified as blind by the breeder, but all were used for mating at 1 or 2 years of age and then sold or culled. Had these rams remained on the property, it is possible they may have gone on to develop blindness, as most affected sheep did not exhibit signs of visual impairment until 3 years of age or older. A dominant mode of inheritance is therefore considered possible.

An X-linked mode of inheritance was considered unlikely as all affected sheep identified were ewes, whereas in X-linked retinitis pigmentosa, females may be unaffected or frequently express milder retinal degeneration than males (Fahim, Daiger, & Weleber, 2000), although some mutations in the X-linked gene *RPGR* also affect female carriers (Daiger, *et al*., 2007). Non-Mendelian inheritance patterns, including digenic, mitochondrial or compound heterozygosity, have also been reported in cases of retinitis pigmentosa (Hartong, *et al.*, 2006; Anasagasti, *et al.*, 2012), and cannot be excluded in Wiltshire sheep with retinal degeneration, especially as pedigree analysis of affected sheep did not demonstrate readily recognisable Mendelian inheritance patterns. However, defining the causative gene is more difficult for diseases with non-Mendelian inheritance than single gene disorders with Medelian segregation patterns (van Heyningen & Yeyati, 2004), and often requires linkage mapping of a large number of affected individuals.

Homozygosity mapping, which assumes the disease has autosomal recessive inheritance, was performed using DNA from five affected sheep and a normal Wiltshire ewe. Ideally, as the mode of inheritance is currently unknown, a separate analysis of Illumina OvineSNP50 BeadChip results would have been performed to detect heterozygous regions and define chromosomal areas of interest if the disease were to be inherited in an autosomal dominant manner. However, the number of affected animals was too low to enable such an analysis to be conducted, as many consecutive heterozygous SNP loci would be shared by the small number of affected sheep. In addition, it would be useful to include the dams and sires of affected animals in such an analysis to help narrow down potential candidate regions, and the parents of all five sheep had been culled so DNA could not be obtained.

Homozygosity mapping detected an identical by descent region in the five affected sheep, consisting of 45 consecutive homozygous SNP loci on chromosome 5. None of the genes within this region are involved in the phototransduction cascade or reported to be specifically expressed in the retina, but the *GPR98* gene located adjacent to this region is of potential interest as a candidate gene. This gene encodes G protein-coupled receptor 98, also known as *VLGR1* (very large G protein-coupled receptor 1) or *MASS1* (monogenic audiogenic seizure susceptibility). G protein-coupled receptors are the largest family of membrane proteins and all have 7 transmembrane domains with alternating intracellular and extracellular loops (Rosenbaum, Rasmussen, & Kobilka, 2009). In vertebrates, G protein-coupled receptors are divided into 5 families, including rhodopsin (family A), secretin (family B), glutamate (family C), adhesion, and Frizzled/Taste2; GPR98 is part of the secretin family, whereas rhodopsin is the prototype for members of family A (Harmar, 2001; Rosenbaum, *et al.*, 2009). *GPR98* is

expressed in most adult tissues, and isoform B of GPR98 is the mammalian ortholog of avian ankle link antigen, which is associated with the stereocilia of the inner ear and the ciliary calyx of photoreceptors of the eye (McMillan & White, 2010). Mutations in *GPR98* in humans have been associated with Usher syndrome type 2C, which is characterised by congenital moderate hearing loss, normal vestibular function and late onset retinitis pigmentosa (Besnard *et al.*, 2012; Yang *et al.*, 2012). The hearing loss typically affects higher frequencies, with affected patients exhibiting a downward-sloping audiogram, and is often not detected until children show delays in language development, with a median age of diagnosis of 8.1 years (Abadie *et al*., 2012). Vision loss usually begins in the second decade of life and is typical of retinitis pigmentosa, with early night and peripheral vision loss, and progressive visual impairment over time (Besnard, *et al*., 2012; Yang, *et al*., 2012). Usher syndrome type 2C is inherited in an autosomal recessive manner (Keats & Lentz, 1999).

As with other forms of retinitis pigmentosa, the retinal pathology of Usher syndrome type 2C includes rod photoreceptor dysfunction, accompanied by milder cone dysfunction, and thinning of the outer nuclear layer of the retina (Schwartz *et al*., 2005). This is similar to the retinal degeneration in Wiltshire sheep, as in affected sheep rod dysfunction predominated over cone dysfunction, leading to the development of night blindness, and the outer nuclear layer was reduced to absent on histological examination of the retina. However, affected Wiltshire sheep showed no appreciable hearing loss, whereas congenital sensorineural hearing loss is a consistent feature in humans with Usher syndrome type 2C (Abadie, *et al*., 2012). Hearing was not specifically assessed in sheep with retinal degeneration, as the possibility of Usher syndrome was only considered after homozygosity mapping had been completed. Affected sheep used

auditory cues (bleating) to help them locate their lambs following separation, which suggests they were able to hear other sheep vocalising nearby, but it was not possible to determine whether there was any degree of hearing impairment based on general observations alone. In addition, Usher syndrome affects hearing at high frequencies more than low frequencies, and sheep naturally have superior auditory acuity at high frequencies compared to humans, so mild hearing loss at higher frequencies may be difficult to detect clinically in sheep (Wollack, 1943; Abadie*, et al.*, 2012).

While *GPR98* could be considered a possible candidate gene for retinal degeneration in Wiltshire sheep based on the similar retinal pathology, it is important to note that Usher syndrome has not been diagnosed in ruminants before and animal models of the disease are limited to transgenic mice. The VLGR1/del7TM mouse expresses a mutant GPR98 protein, with deleted transmembrane and cytoplasmic domains, and although hearing loss is similar to what is described in people with Usher syndrome type 2C, the retinal phenotype is not replicated in this model (Williams, 2008). In VLGR1/del7TM mice, the retina usually has a normal fundoscopic and histological appearance, but there is age-related loss of visual function detectable on electroretinography at 15 months of age, which is not apparent in wild-type mice. This relative lack of retinal pathology in mutant mice may be due to poorly developed calycal processes in mice compared to other species (McGee *et al*., 2006). Calycal processes are microvillus-like structures which cup the base of the outer segment of rods and cones, and are the site of localisation of the ankle link antigen complex/GPR98 (Dosé *et al*., 2003; Yang, 2012). Specific research regarding the development of calycal processes in sheep is lacking, so it is not possible to speculate on whether they would exhibit retinitis pigmentosa as part of Usher syndrome, although amphibians (Fetter &

Corless, 1987; Wolfrum, 2011), fish (Collin, Collin, & Ali, 1996), chicks (Goodyear & Richardson, 1999), pigs (Gloesmann *et al*., 2003) and primates (Rana & Taraszka, 1991) have been shown to have prominent calycal processes. Alternatively, the relatively short lifespan of mice may mean that they do not live long enough to develop retinal degeneration, as blindness in humans with Usher syndrome usually has a late-onset (Schwartz*, et al.*, 2005; McGee*, et al.*, 2006).

To further investigate *GPR98* as a candidate gene for retinal degeneration in Wiltshire sheep, it would be necessary to sequence this gene in affected individuals. However, this is a difficult undertaking as the gene is extremely large and spans from position 87499346 to 88022860 on chromosome 5 in sheep. In humans, it is a 90 exon gene encoded by an 18.9kb open reading frame, and the primary translation product is approximately 700kDa, making it the largest cell surface protein known (Ebermann *et al*., 2009; McMillan & White, 2010). Mutations in *GPR98* in humans with Usher syndrome have been found throughout the sequence, and all possible types of DNA alterations have been reported, including substitutions, small and large duplications and deletions, splicing alterations of pre-mRNA caused by intronic or exonic mutations, creation of premature stop codons and missense mutations. Most of these mutations lead to the formation of a truncated protein product (Besnard, *et al*., 2012), and the clinical presentation appears to be similar regardless of which particular mutation is responsible (Hilgert *et al*., 2009). Due to the large size of *GPR98*, it is not routinely screened in patients with Usher syndrome, but it is hoped that advances in next generation sequencing of whole exomes will make further investigation of *GPR98* more cost-effective and less labour intensive (Besnard, *et al*., 2012).

3.4.1 SUMMARY

Rhodopsin was chosen as a candidate gene in this study on the basis of its central role in the phototransduction cascade and involvement in a high proportion of cases of retinitis pigmentosa. Sequencing the exonic regions of the rhodopsin gene in Wiltshire sheep with progressive retinal degeneration did not reveal any significant mutations, but the presence of intronic or quantitative mutations within this gene cannot be excluded. To identify chromosomal regions identical by descent, homozygosity mapping was performed using the Illumina OvineSNP50 BeadChip and affected Wiltshire sheep were homozygous for 45 consecutive SNP loci on chromosome 5. The *GPR98* gene is located adjacent to this region, and mutations in this gene are a cause of Usher syndrome type 2C in humans, characterised by sensorineural hearing loss and late-onset retinal degeneration. While *GPR98* could be a suitable candidate gene for retinal degeneration in Wiltshire sheep, further work to establish the probable mode of inheritance of this disease is required before undertaking more candidate gene studies, as pedigree analysis of affected sheep did not convincingly support any particular inheritance pattern.

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Chapter 4

GENERAL DISCUSSION

4.1 INTRODUCTION

The central aim of this research was to describe and investigate adult-onset blindness identified in a single flock of Wiltshire sheep during 2011 and 2012. Particular emphasis was placed upon describing the clinical, ophthalmic and histological features of the disease, as well as investigating the molecular genetics of the disease. The results of the studies undertaken confirm progressive retinal degeneration primarily targeting rod photoreceptors, with a probable inherited basis in purebred Wiltshire sheep. Inherited retinal degenerations affecting rod photoreceptors have not been previously reported in sheep, but the disease has similarities to retinitis pigmentosa in humans and progressive retinal atrophy in dogs.

4.2 FEATURES & DIAGNOSIS OF RETINAL DEGENERATION IN WILTSHIRE SHEEP

Affected Wiltshire sheep had apparently normal vision at birth and did not show any signs of visual impairment until they were 2 to 3 years old. At this age, affected sheep showed signs of reduced vision in low ambient light conditions, but appeared to have normal vision during bright daylight. Significant ophthalmic examination findings were limited to the retina, and in the early stages of the disease there was bilateral generalised patchy tapetal hyperreflectivity, indicating thinning of the retina. Mild attenuation of retinal blood vessels was also evident. Histologically, it could be seen that the thinning of the retina was due to severe loss of rod photoreceptors, with only a single layer of cone photoreceptors remaining. Rod photoreceptors are primarily responsible for night-time (scotopic) vision (Bloomfield & Dacheux, 2001), which explains why the sheep initially developed night blindness. Retinal degeneration progressed over time, and 4 to 6 year old affected sheep appeared blind in bright daylight as well as in low light conditions. These older sheep had generalised tapetal hyperreflectivity, severe retinal blood vessel attenuation and the optic disc appeared pale on ophthalmological exam. Histology of the retina of a 6 year old ewe (43-06) showed almost complete loss of both rod and cone photoreceptors, with attenuation of the outer plexiform layer. Vision loss was not accompanied by neurological signs in any sheep, and histological examination of the brains of three sheep showed no significant abnormalities.

Identification of affected sheep was more difficult than anticipated, as sheep are able to compensate well for visual impairment in a flock situation. Maze testing in low light conditions was an effective method of detecting sheep with early retinal

degeneration, but the short period of suitable ambient light conditions at dusk meant that sheep had to be run through the maze in groups of three, with performance assessed subjectively. Ideally, to increase the sensitivity of detection of early retinal degeneration, all sheep would have been subjected to a fundic examination, but this would be very time-intensive and for this reason it would not be a suitable screening tool in larger flocks.

4.3 SIMILARITIES TO OTHER RETINAL DISEASES IN SHEEP

The initial presentation of retinal degeneration in Wiltshire sheep is similar to that reported with bright blindness in sheep, which is due to chronic ingestion of bracken fern containing the toxic principle ptaquiloside (Hirono *et al*., 1993). Sheep with bright blindness are typically 3 to 4 years old and have narrowing of retinal blood vessels and increased tapetal reflectivity on ophthalmic examination (Watson, Barnett, & Terlecki, 1972), which are also features of the retinal degeneration in Wiltshire sheep. However, the two diseases differ histologically, as in bright blindness there is swelling and fragmentation of both rod and cone photoreceptors, particularly cones, whereas in the disease in Wiltshire sheep there is significant loss of rod photoreceptors with relative sparing of cones. In addition, affected Wiltshire sheep had never been exposed to bracken fern, and bright blindness has not been reported previously in New Zealand (Smith, 1990).

Other retinal diseases reported in sheep include closantel toxicity (Gill *et al*., 1999), ocular toxoplasmosis (Piper, Cole, & Shadduck, 1970), vitamin A deficiency (Moore, 1939), ceroid lipofuscinosis (Graydon & Jolly, 1984), and other plant toxicities (Main *et al.*, 1981; Lugt, Olivier, & Jordain, 1996). The features of retinal degeneration

in Wiltshire sheep were not compatible with any of these diseases, particularly as most also affect the central nervous system, whereas lesions are confined solely to the retina in Wiltshire sheep.

Pedigree analysis of affected Wiltshire sheep supported an inherited basis for the disease. Inherited retinopathies are rare in sheep and, to the author's knowledge, day blindness in Awassi sheep is the only inherited primary retinal disease reported in sheep to date. In this disease, cone dysfunction is detectable in young lambs, but normal retinal histology is preserved (Shamir *et al*., 2010), in contrast to the disease in Wiltshire sheep where severe loss of rod photoreceptors is observed histologically.

4.4 SIMILARITIES TO RETINAL DISEASES IN OTHER SPECIES

The retinal degeneration in Wiltshire sheep shares similarities with progressive retinal atrophy (PRA) in dogs and retinitis pigmentosa (RP) in humans. Both PRA and RP are characterised by initial degeneration of rod photoreceptors, resulting in night blindness (Hartong, Berson, & Dryja, 2006; Miyadera, Acland, & Aguirre, 2012). Vision loss progresses over time, and there is usually delayed or secondary loss of cone photoreceptors, which eventually results in complete blindness. The retinal degeneration in Wiltshire sheep follows a similar clinical course, and histology of affected sheep shows rod photoreceptors are initially targeted, with cones lost secondarily. Fundic examination findings are also comparable between PRA, RP and affected Wiltshire sheep, with the exception of tapetal hyperreflectivity, which is not observed in patients with RP as humans do not have a tapetum.

Both PRA and RP are genetically and phenotypically diverse diseases, with mutations in 18 genes identified as causes of PRA in dogs (Miyadera, *et al*., 2012), and mutations in 50 genes currently associated with RP in humans (Daiger, 2014). Most cases of PRA are inherited in an autosomal recessive manner, while in RP the proportions of autosomal dominant and autosomal recessive inheritance are similar, with a smaller number of cases exhibiting X-linked or digenic inheritance (Fahim, Daiger, & Weleber, 2000; Daiger, Bowne, & Sullivan, 2007). Pedigree analysis of Wiltshire sheep with retinal degeneration failed to provide clear evidence for either autosomal dominant or autosomal recessive inheritance, although autosomal dominant inheritance was suspected. Determination of the probable mode of inheritance was complicated by the likelihood that a significant number of sheep may have been affected over recent years, but not identified as such, either because they were sold at an early age (before detectable retinal degeneration could develop), or had subtle signs of visual impairment that were not readily apparent in an extensive farming situation.

4.5 MOLECULAR GENETICS

Initial attempts to identify the molecular basis of retinal degeneration in Wiltshire sheep have been unsuccessful. At the outset of this study, a comparative candidate gene approach was used as there were only three ewes with confirmed retinal degeneration available for genetic analysis, which is insufficient for homozygosity and heterozygosity mapping. Rhodopsin was chosen as a candidate gene due to the central role it plays in the phototransduction cascade of vision (Pepe, 1999), and its involvement in a relatively high proportion of retinitis pigmentosa cases in humans, particularly autosomal dominant retinitis pigmentosa (Daiger, *et al*., 2007). Sequencing targeted exonic regions, as almost all mutations in rhodopsin in humans result in

changes or deletions of one or a few amino acids, leading to the synthesis of an abnormal protein (Anasagasti *et al.*, 2012). No disease-associated mutations were found in rhodopsin in the three affected sheep used for sequencing, but the possibilities of an intronic mutation or a mutation causing a quantitative change in rhodopsin synthesis cannot be excluded.

Two further affected sheep were identified on the second visit to the property in June 2012, giving a total of five affected sheep available for genetic analysis. This number was considered sufficient for homozygosity mapping, which assumes the disease is inherited in an autosomal recessive manner. Analysis of results from the Illumina OvineSNP50 BeadChip showed the five affected sheep, but not a normal Wiltshire sheep, had an identical by descent region spanning 45 consecutive SNP loci on chromosome 5. No genes within this region were known to be specifically expressed in the retina, but the *GPR98* gene, which encodes G-protein receptor 98, lies immediately adjacent to this region. Mutations in *GPR98* are associated with Usher syndrome type 2C in humans, characterised by late-onset retinitis pigmentosa and sensorineural hearing loss, inherited in an autosomal recessive manner (Schwartz *et al*., 2005). Retinal changes in the Wiltshire sheep are similar to those described in retinitis pigmentosa of Usher syndrome, but affected sheep do not show any clinical signs of hearing loss. No specific assessments of hearing were undertaken in the sheep, so it is possible that mild hearing loss may have gone undetected. Therefore, in order for *GPR98* to be considered as a potential candidate gene, further investigation into the hearing of affected sheep would be required, such as measurement of brainstem auditory evoked responses, a method which has been successfully utilised in sheep

previously (Griffiths *et al.*, 1996). Additionally, more work to determine the probable mode of inheritance is required before pursuing further candidate gene studies.

4.6 STUDY LIMITATIONS

A major limitation of the present study is the low number of affected sheep available for molecular genetic investigations. As alluded to in previous paragraphs, there were only five sheep on the property with confirmed retinal degeneration at the time this research was undertaken. Four additional sheep had been diagnosed with retinal degeneration by a certified veterinary ophthalmologist prior to the start of this study (giving a total of nine affected animals), but these sheep had been culled by the time the study began and blood was not collected for DNA extraction. Histology was performed on the eyes of two of these four sheep, and findings confirmed retinal degeneration targeting rod photoreceptors, as seen in the other affected sheep.

It is thought that the low number of affected Wiltshire sheep is a consequence of the late onset of the disease and difficulty in detecting visual impairment in an extensive farming system, rather than a truly low prevalence of disease. As discussed previously, sheep with retinal degeneration did not show evidence of visual impairment until 2 to 3 years of age, and even then, signs were very subtle unless sheep were subjected to a maze challenge in low light conditions. This potential under-diagnosis, particularly of sheep which are sold or culled before 3 years of age, may have been one reason why pedigree analysis to determine the likely mode of inheritance was inconclusive. Additionally, the disease may have been present undetected in other Wiltshire flocks, particularly larger flocks, as the subtle signs of night blindness and progressive visual impairment could be easily overlooked unless each sheep is observed individually.

To further investigate potential candidate genes, it would have been desirable to analyse the results of the Illumina OvineSNP50 BeadChip to define heterozygous regions in affected sheep, which could be of interest if the disease is inherited in an autosomal dominant manner. This was not done as part of the current study, as five sheep are likely to share too many heterozygous loci to effectively identify chromosomal regions of interest. Also, it is desirable to include dams or sires of affected sheep as BeadChip subjects, as this can help to refine loci of interest, but no DNA was available from the parents of affected sheep.

Histological studies indicate there is a primary loss of rod photoreceptors in this disease, with secondary loss of cones. To confirm and further characterise the degeneration of rods, it may be useful to perform electron microscopy on affected sheep retinas. Electron microscopy has been performed in limited cases of retinitis pigmentosa in humans (Kolb & Gouras, 1974; Flannery *et al.*, 1989), where it has allowed detailed comparison of the degenerative processes occurring in different regions of the retina and different retinal cell types. Studies investigating the pathology of retinitis pigmentosa have also used confocal microscopy and immunocytochemistry to further characterise the disease (Li, Jacobson, & Milam, 1994; Fariss, Li, & Milam, 2000).

4.7 FUTURE DIRECTIONS

In late 2013 and early 2014, three additional Wiltshire sheep were identified with suspected retinal degeneration. These three sheep were all born on the same farm as the rest of the affected sheep, but one of them (204-08) is a ram that had been sold to a local client, who noticed he had become blind. As yet, there are no reports of blindness in the

flock where this ram was used, but no maze testing or ophthalmic examinations have been performed on sheep on this farm to date. The other two sheep suspected to have developed retinal degeneration are 3 year old ewes (7-10 and 11-10) that have remained as part of the original flock, and both are daughters of ewes known to be blind (71-07 and 43-06 respectively). The pedigrees of these recently identified blind sheep are included in Appendix (Figure A.2).

The breeder reports that these three sheep show definite signs of visual impairment and are unable to navigate their way through the yards at dusk. The age of onset and clinical presentation of blindness is identical to that described in other affected sheep, but as visual impairment was only detected very recently, retinal degeneration has not yet been confirmed on fundic examination. Planning is underway to transport these three sheep to Palmerston North, where a full ophthalmic examination and retinal photography can be performed by the primary investigator. If retinal degeneration is confirmed, it is hoped that these sheep can be used as part of a small breeding trial to help confirm the mode of inheritance of this disease in Wiltshire sheep. The three recently identified blind sheep provide further evidence to support a dominant mode of inheritance, as the two ewes are both daughters of affected ewes, and with dominant inheritance every affected animal is expected to have an affected parent (Nicholas, 1987).

If a flock of Wiltshire sheep with retinal degeneration can be established in Palmerston North, this will create opportunities to investigate additional aspects of the disease, particularly changes which occur early in the course of the disease prior to the development of detectable visual impairment. In humans, electroretinography can be a

useful tool in the diagnosis of retinitis pigmentosa early in life, before significant fundic abnormalities are appreciable (Berson, 1987). Electroretinography has been previously used in sheep to characterise the progression of retinal degeneration associated with ceroid lipofuscinosis in South Hampshire lambs (Graydon & Jolly, 1984), and if performed in affected Wiltshire sheep, it could provide further information about the relative involvement of rod and cone photoreceptors in this disease, and how photoreceptor degeneration progresses over time.

Based on the pattern of occurrence of the disease to date, it is expected that additional cases of retinal degeneration in Wiltshire sheep will be identified over time, as affected sheep reach an age when detectable retinal changes or visual impairment develop. Furthermore, due to the relatively narrow genetic pool of pure Wiltshire sheep in New Zealand (Morrison Farming), it is possible that the disease is present undetected in other Wiltshire flocks, and increasing awareness of it amongst Wiltshire breeders may result in further cases being identified. Alternatively, the mutation could also have been introduced from another breed, as polled Wiltshire sheep originate from crossing Wiltshire Horn sheep with a polled breed, then back crossing the progeny to Wiltshire Horn sheep for four generations. As the number of affected animals available for genetic analysis increases, further mapping using the Illumina OvineSNP50 BeadChip to identify heterozygous regions in affected sheep may become practical, and this could aid in selecting candidate genes for investigation if inheritance proves to be dominant. Genes which are part of the phototransduction cascade are of particular interest as possible candidate genes, including transducin *(GNAT1),* which is inherited in an autosomal dominant manner (Phelan & Bok, 2000), and the α and β phosphodiesterase subunits (*PDE6A* and *PDE6B)*, which are implicated in some recessively inherited

retinal degenerations (Hartong, *et al*., 2006). Whole genome sequencing of affected sheep is also being considered as an alternative approach to investigating the genetic basis of retinal degeneration in Wiltshire sheep.

If further attempts to characterise the molecular basis of the disease are successful, it is possible that the retinal degeneration in Wiltshire sheep could be a suitable model for the analogous form of retinitis pigmentosa in humans. Large animal models of retinitis pigmentosa are currently limited to transgenic pigs expressing mutant rhodopsin (Li *et al*., 1998), although for some mutations there are suitable small animal models in mice, rats, cats, dogs and chickens (Voaden, 1991). Animal models are important for understanding the pathophysiology of retinal degenerations and in the development and testing of treatment regimens (Chader, 2002).

4.8 CONCLUSIONS

The results of the current research confirm a novel retinal degeneration targeting rod photoreceptors in Wiltshire sheep. Affected sheep develop detectable night blindness at 2 to 3 years of age and vision loss is progressive over time, resulting in complete blindness by 4 to 6 years of age. The disease appears to be inherited in an autosomal dominant manner, and has similarities to progressive retinal atrophy in dogs and retinitis pigmentosa in humans. Preliminary studies in affected sheep did not identify any significant mutations in the rhodopsin gene, which is implicated in some cases of progressive retinal atrophy and retinitis pigmentosa, although a wide array of other genes are also associated with these diseases. Further research is planned to confirm the probable mode of inheritance and investigate the underlying molecular basis of this disease in Wiltshire sheep.

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APPENDIX

***Table A.1*: Selected genes for retinitis pigmentosa in humans, their mode of inheritance, and the location of the analogous gene in the sheep (OAR_v3.1)**

		Inheritance	Sheep chromosome
Phototransduction cascade			
RHO	rhodopsin (G-protein coupled photon receptor)	Dominant, recessive	**19** 56085881..56092120
PDE6A	rod cGMP-phosphodiesterase α subunit (G-protein effector enzyme)	Recessive	**5** 58707215..58788055
PDE6B	rod cGMP-phosphodiesterase β subunit (G-protein effector enzyme)	Recessive	**6** 116901725..116929069
GNAT1	guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1 (transducin)	Dominant	**19** 50105494..50108868
CNGA1	rod cGMP-gated cation channel α subunit	Recessive	**6** 66646169..66686867
CNGB1	rod cGMP-gated cation channel β subunit	Recessive	**14** 25240413..25307264
SAG	arrestin (rhodopsin deactivation)	Recessive	**1** 7415905..7449421
Vitamin A metabolism			
ABCA4	ATP-binding cassette protein A4 (photoreceptor disc membrane flippase for vitamin A)	Recessive	**1** 70306557..70456182
RLBP1	retinaldehyde binding protein (11-cis-retinaldehyde carrier)	Recessive	**18** 19813457..19824798
RPE65	(vitamin A trans-cis isomerase)	Recessive	**1** 43721797..43745688
LRAT	lecithin retinol acetyltransferase (synthesises vitamin A esters)	Recessive	**17** 2473080..2473974
RGR	RPE-vitamin A G-protein coupled receptor (photon receptor in RPE)	Recessive	**25** 38843805..38853400
Structural or cytoskeletal			
RDS/ PRPH2	peripherin (outer disc segment membrane protein)	Dominant, digneic	**20** 16465543..16479760
ROM1	rod outer segment protein	Digenic	**21** 40343792..40345259
FSCN2	fascin (actin bundling protein)	Dominant	**11** 50397859..50403453
TULP1	tubby-like protein 1	Recessive	**20** 9574110..9588018
CRB1	crumbs homologue (transmembrane protein adherent junctions)	Recessive	**12** 74335751..74515404
RP1	microtubule-associated protein (microtubule formation and stabilisation)	Dominant, recessive	**9** 35170933..35180109

(continues on next page)

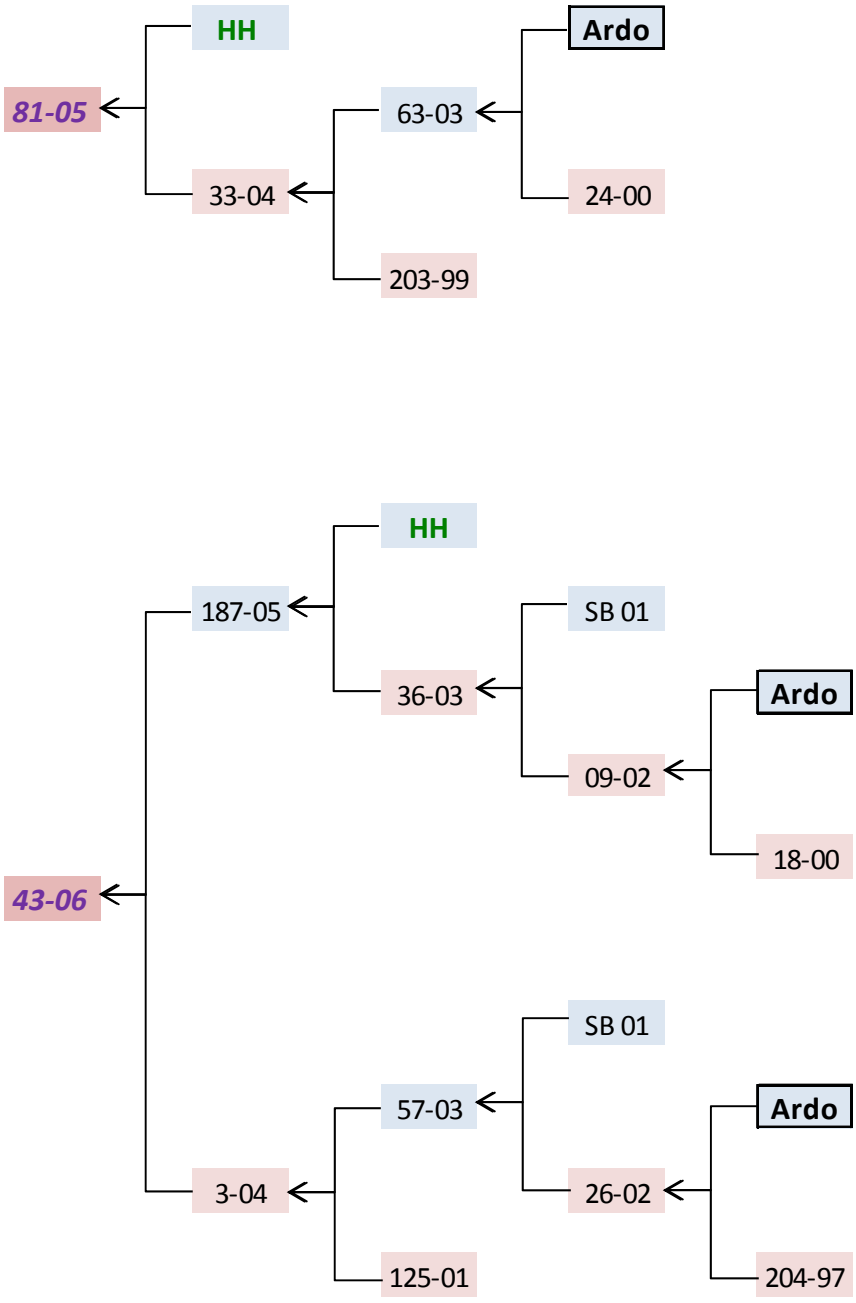
Signalling, cell-cell interaction, or synaptic interaction			
SEMA4A	semaphoring B transmembrane immune system protein	Dominant	**1** 104748805..104767309
CDH23	cadherin 23 (adhesion receptor)	Recessive	**25** 27415341..27733985
PCDH15	protocadherin 15 (adhesion receptor)	Recessive	**22** 4404770..5313981
USH1C	Usher's syndrome type 1C (integrating scaff old protein harmonin)	Recessive	**15** 34542154..34578930
USH2A	Usher's syndrome type IIA (Usher's network protein)	Recessive	**12** 16999001..17940700
MASS1	monogenic audiogenic seizure susceptibility 1 (Usher's network protein)	Recessive	
USH3A	Usher's syndrome type IIIA (transmembrane protein clarin 1)	Recessive	
RP2	plasma membrane associated protein	X-linked	**X** 54477862..54530310
RNA intron-splicing factors			
PRPF31	precursor mRNA-processing factor 31 (spliceosome component)	Dominant	**14** 60325446..60335465
PRPF8	precursor mRNA-processing factor 8 (spliceosome component)	Dominant	**11** 22375692..22406577
PRPF3	precursor mRNA-processing factor 3 (spliceosome component)	Dominant	**1** 99304599..99323530
RP9	PIM1-associated protein (RNA splicing factor)	Dominant	**4** 63716874..63728474
Traffic king of intracellular proteins			
MYO7A	myosin 7A (melanosome motility protein)	Recessive	**15** 54677185..54764057
USH1G	scaff old protein containing ankyrin repeats and SAM domain (Usher's type I protein traffic regulator)	Recessive	**11** 55502712..55506180
BBS1	Bardet-Biedl syndrome 1	Recessive	**21** 43856792..43873155
BBS2	Bardet-Biedl syndrome 2	Recessive	**14** 23604985..23635064
ARL6	ADP-ribosylation factor like 6	Recessive	**1** 160513035..160548552
BBS4	Bardet-Biedl syndrome 4	Recessive	**7** 19124327..19170984
BBS5	Bardet-Biedl syndrome 5	Recessive	**2** 138858676..138879165
MKKS	McKusick-Kaufman syndrome	Recessive	**13** 3163556..3175999
BBS7	Bardet-Biedl syndrome 7	Recessive	**6** 3617254..3655934

(continues on next page)

TTC8	tetratricopeptide repeat domain 8	Recessive	**7** 97364743..97424451
PTHB1	parathyroid hormone-responsive B1 gene	Recessive	
RPGR	trafficking of proteins in the cilia	X-linked	**X** 35701365..35762176
pH regulation (choriocapillaris)			
CA4	carbonic anhydrase IV (carbon dioxide/bicarbonate balance)	Dominant	**11** 12378891..12386308
Phagocytosis			
MERTK	mer tyrosine kinase proto-oncogene (RPE receptor involved in outer segment phagocytosis)	Recessive	**3** 105972402..106063574
Other			
CERKL	ceramide kinase-like (ceramide converting enzyme)	Recessive	**2** 127014751..127160854
IMPDH 1	inosine-5' monophosphate dehydrogenase type I (guanine nucleotide synthesis)	Dominant	**4** 92639093..92656126
BBS10	vertebrate-specific chaperonin-like protein	Recessive	**3** 112151866..112154610

Adapted from Hartong, D. T., Berson, E. L., & Dryja, T. P. (2006). Retinitis pigmentosa. *The Lancet, 368*(9549), 1795-1809.

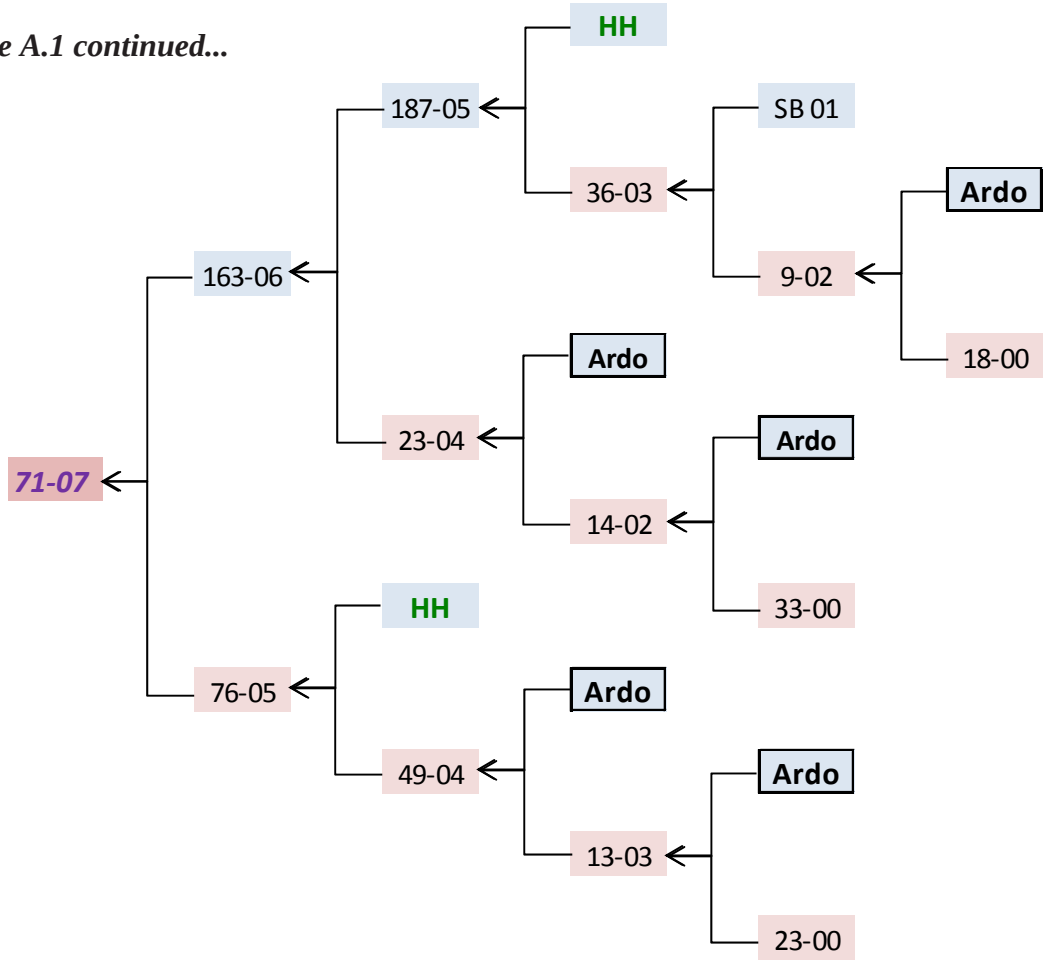
Figure A.1: **Pedigrees of Wiltshire sheep with confirmed retinal degeneration**



Key:

	Male
	Female
Ardo	Ardo 00-394
HH	HH 03-88
AV	AV 624-06
SB 01	SB 01-W7
SB 02	SB 02-08
84-07	Bold & italic = affected sheep

Figure A.1 continued...



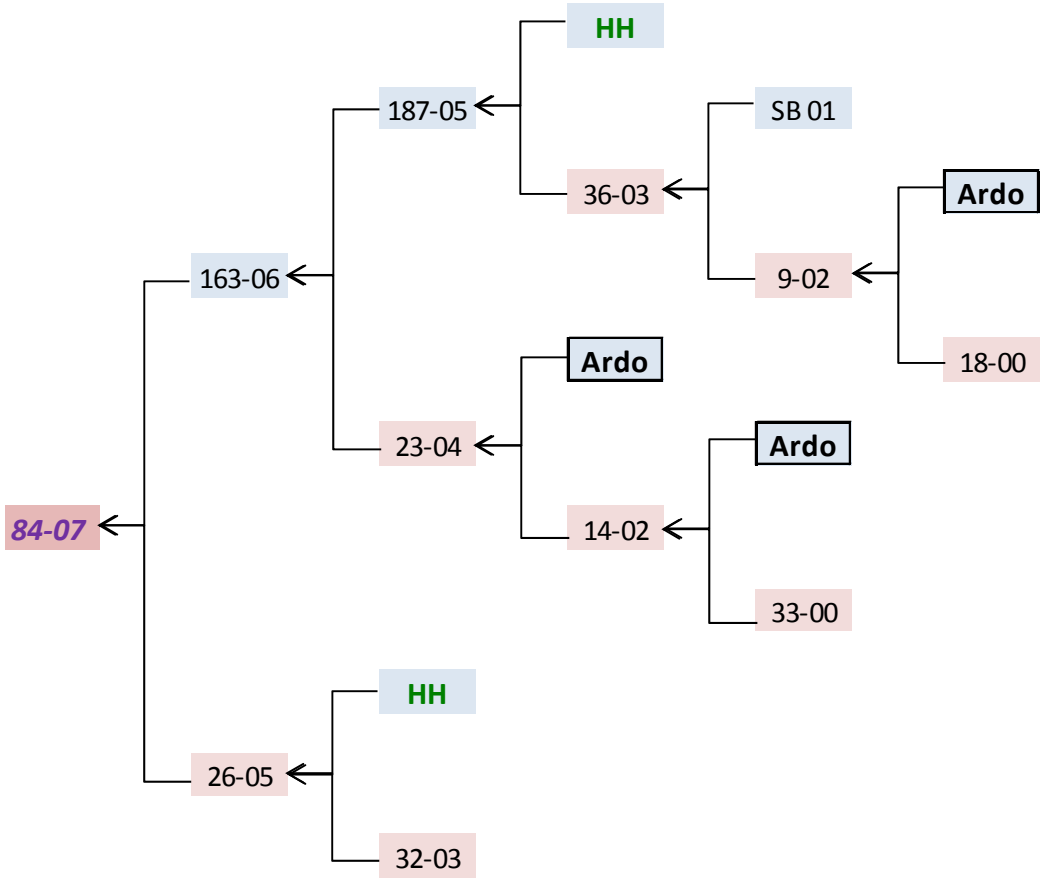
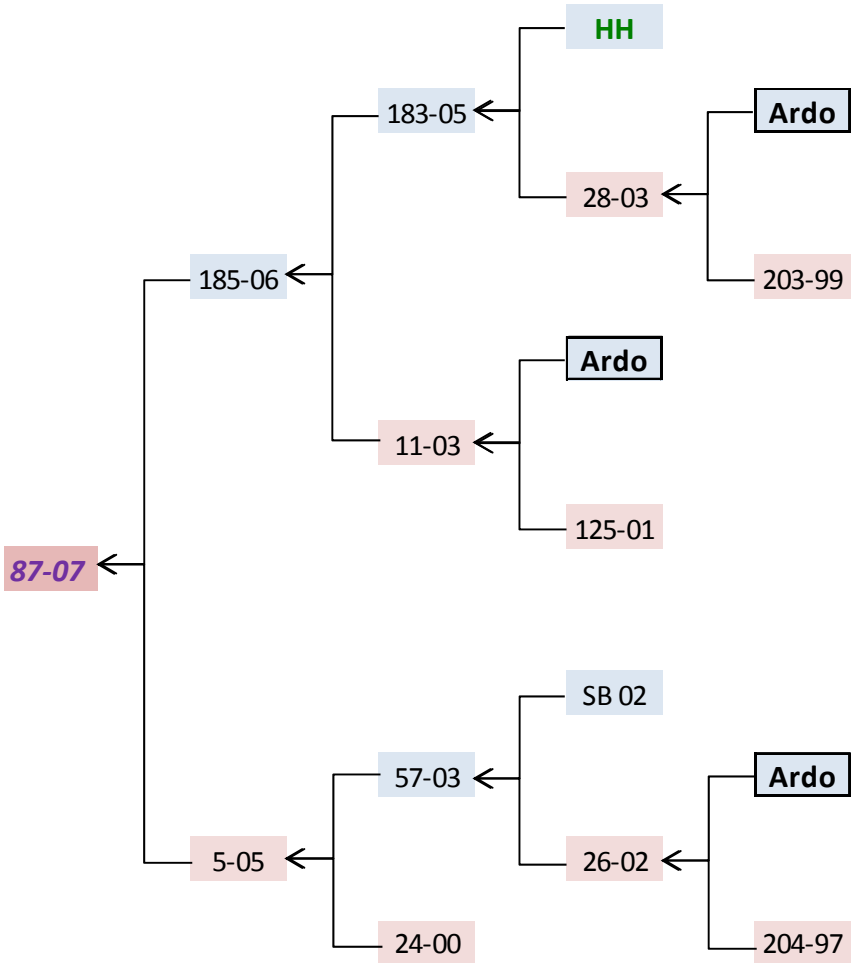


Figure A.1 continued...



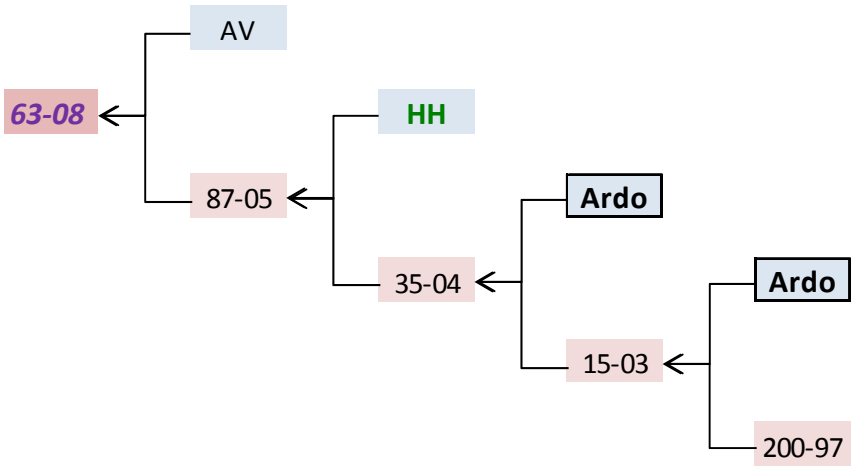
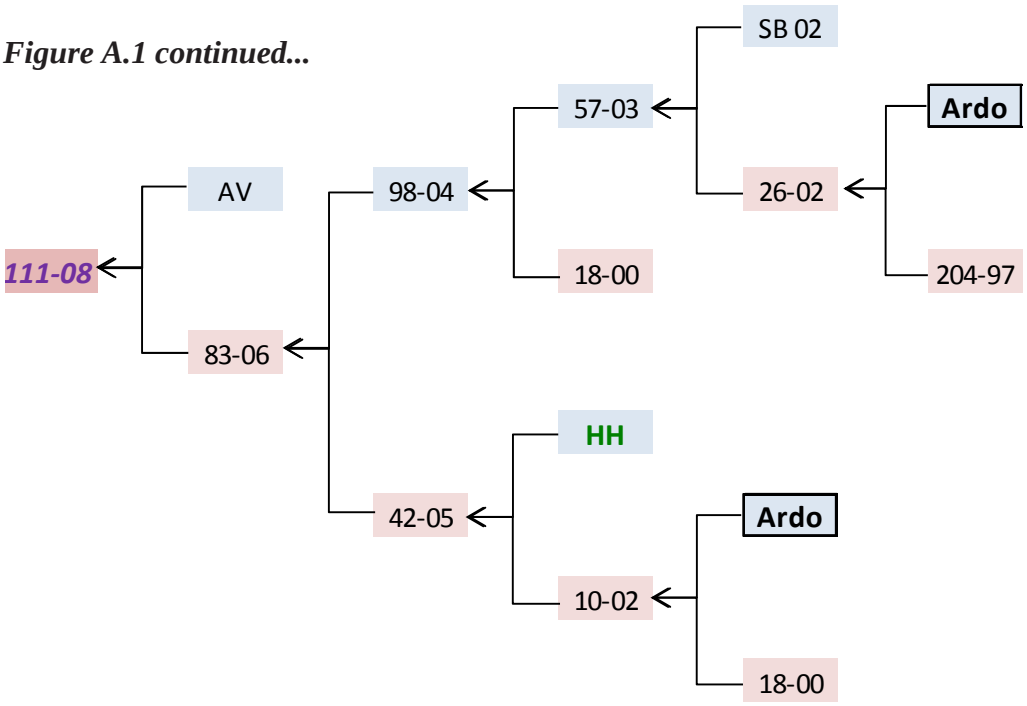


Figure A.1 continued...



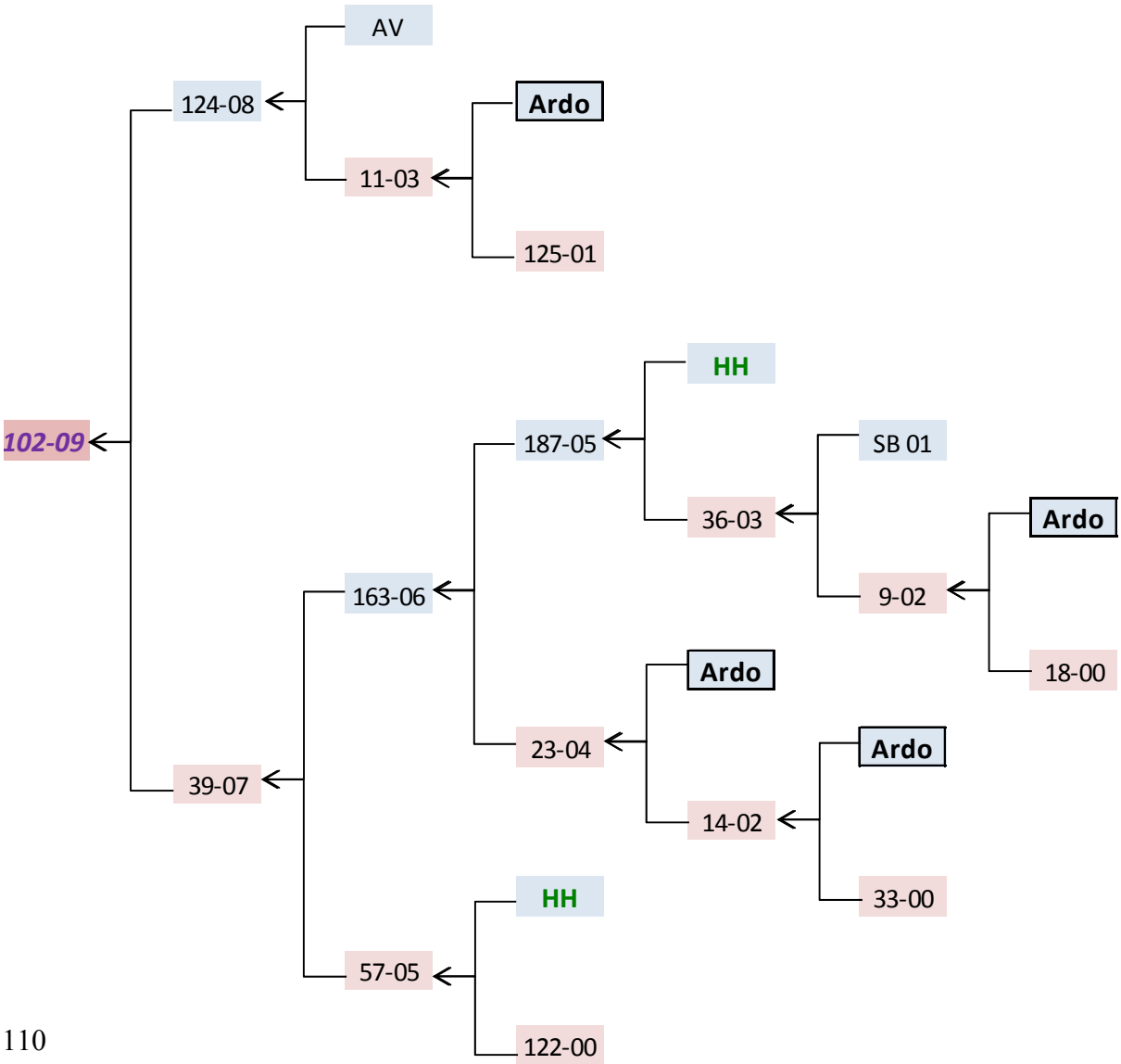


Figure A.1 continued...

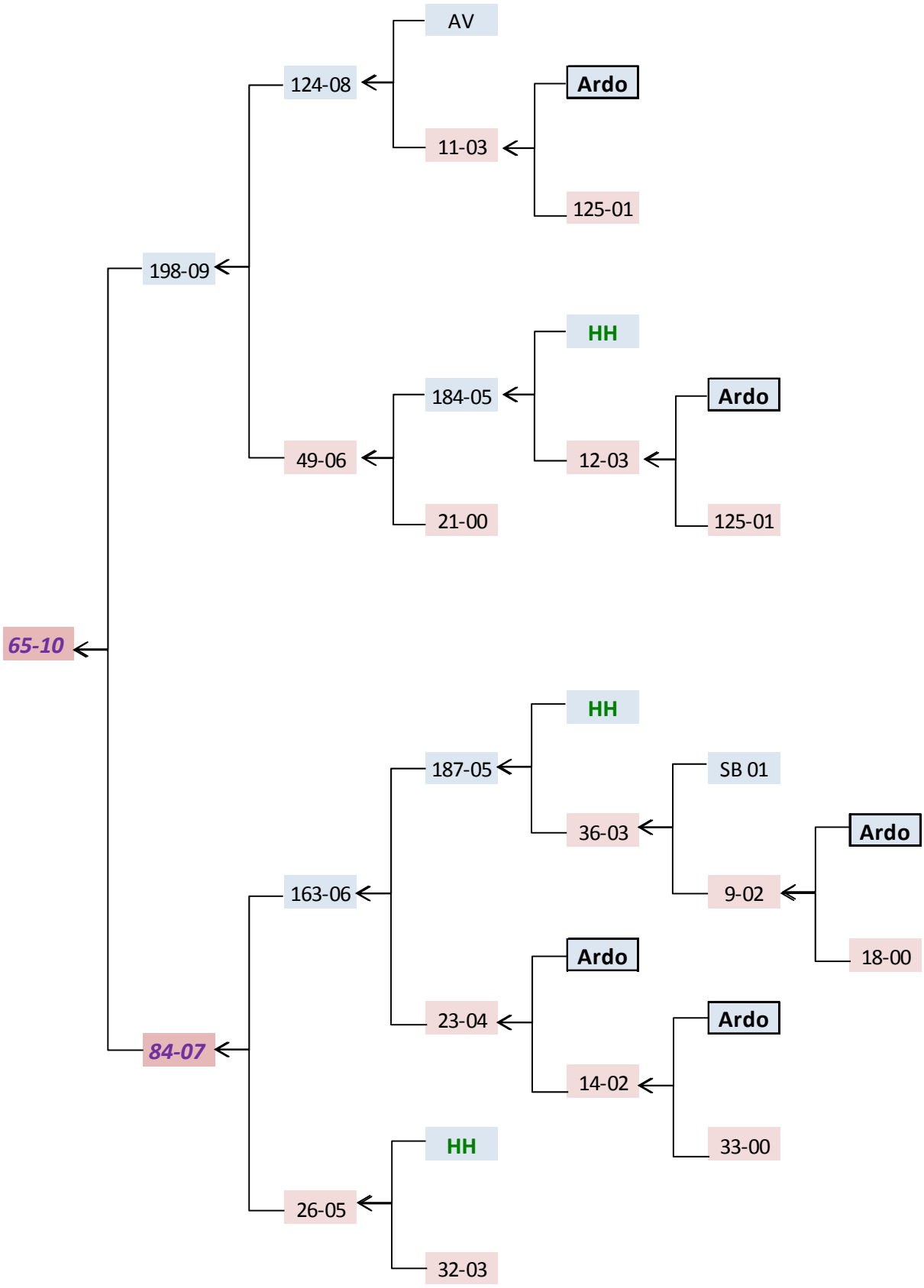


Figure A.2: **Pedigrees of additional Wiltshire sheep with suspected retinal degeneration, identified in January 2014**

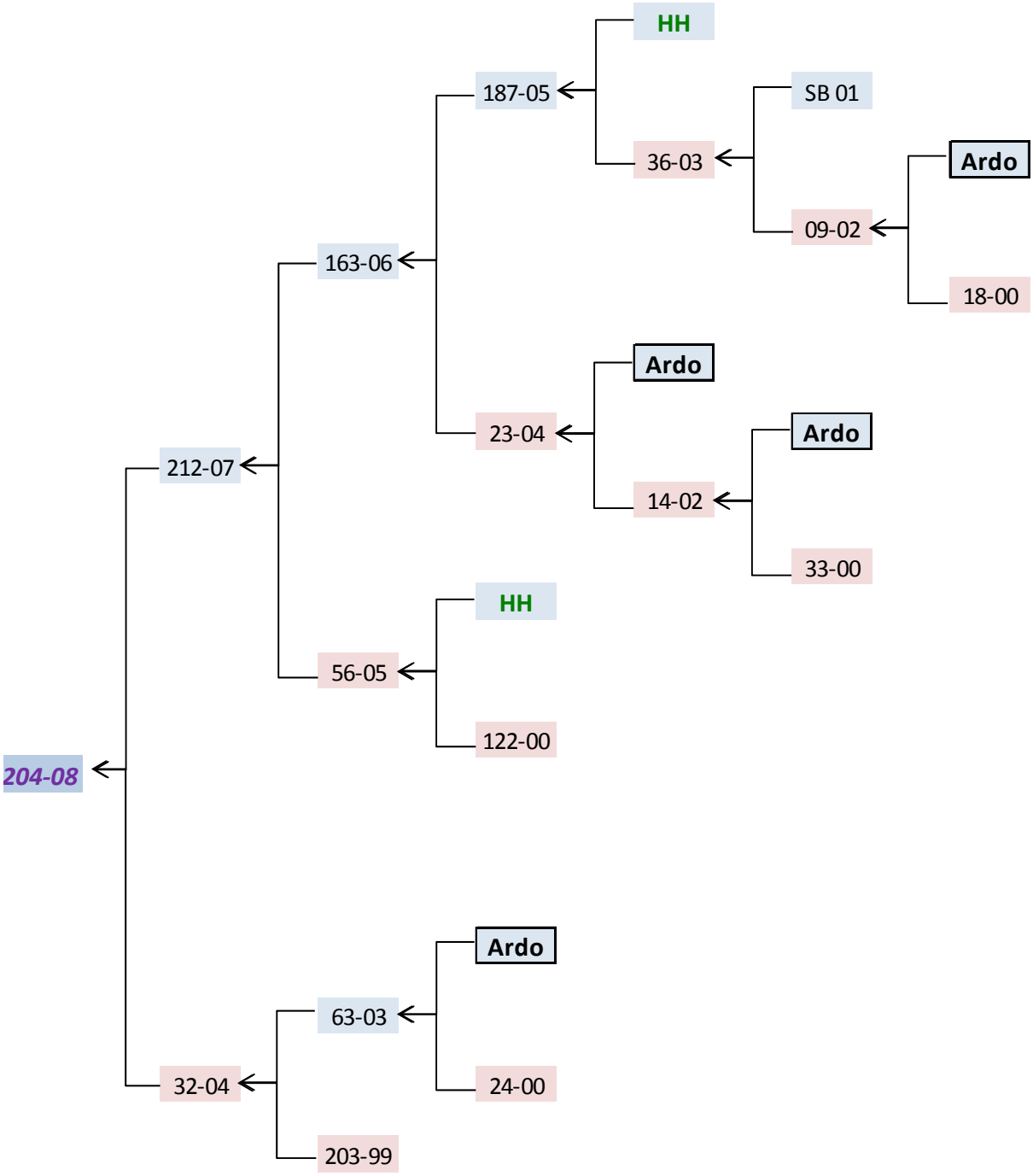


Figure A.2 continued...

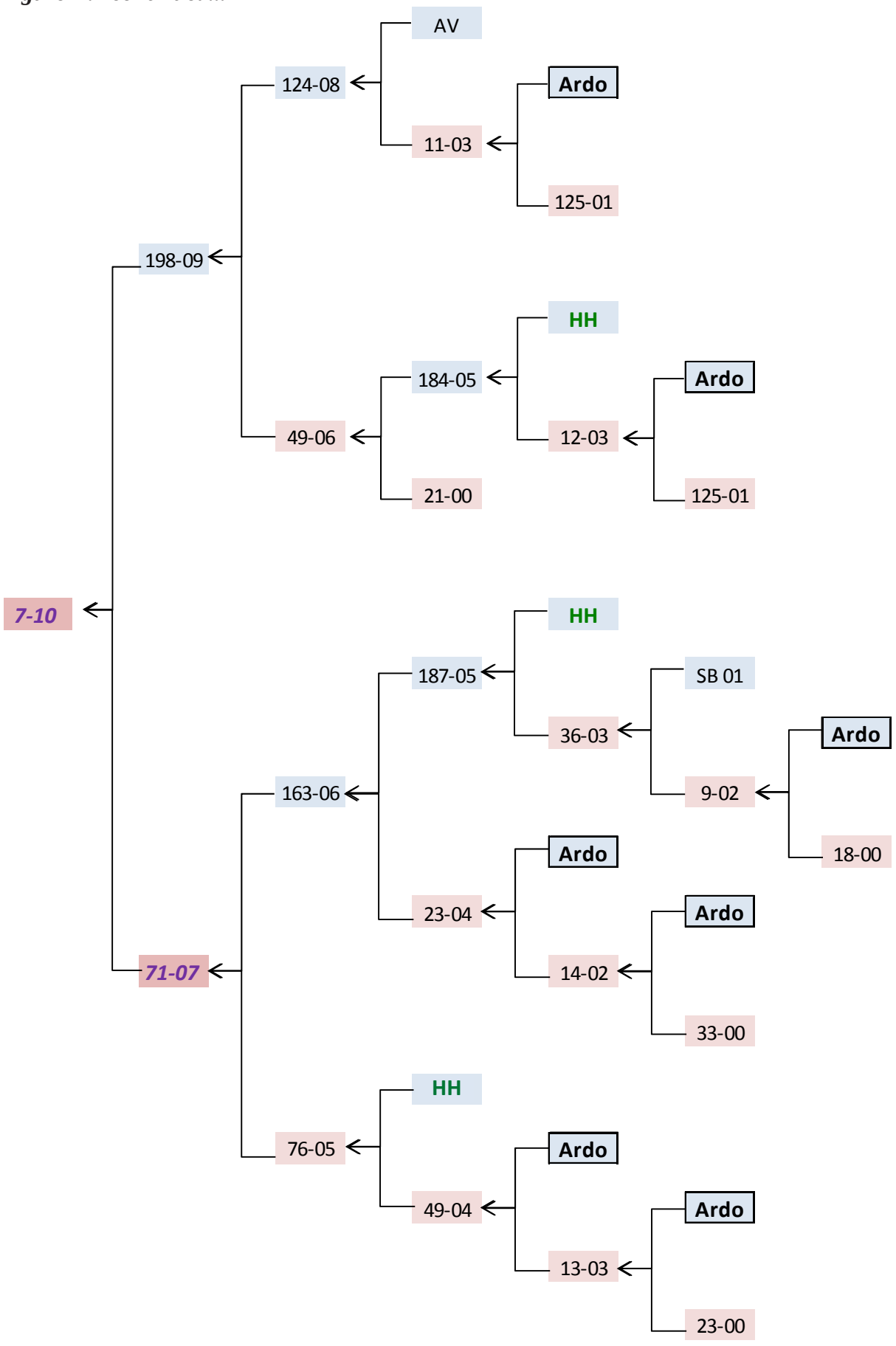


Figure A.2 continued...