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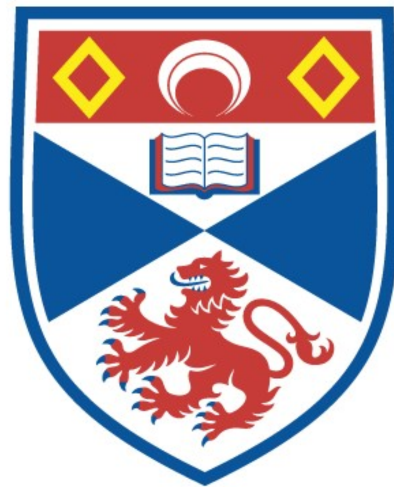

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Genetic analysis of mate choice, offspring survival and pathogen susceptibility in natural populations

Dissertation submitted to the University of St Andrews for the degree of

Doctor of Philosophy

by

KYI BEAN

February, 2005

Th F8

Grey seal (*Halichoerus grypus*) pups

Preface

(i) I, Kyi Bean, hereby certify that this thesis, which is approximately 40,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

21st June 2005

(ii) I was admitted as a research student in February 2002 and as a candidate for the degree of Doctor of Philosophy in February, 2003; the higher study for which this is a record was carried out in the University of St Andrews between 2002 and 2005.

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Summary

Genetic variation has increasingly been shown to be important in determining mating strategy, offspring survival and pathogen susceptibility. Such variation can be general and involve genome-wide measures of heterozygosity such as levels of inbreeding, or can involve specific genes such as those of the major histocompatibility complex (MHC). Despite a number of studies documenting MHC-correlated mate choice and pathogen susceptibility, such studies have largely been restricted to humans and laboratory animals. Thus, the extent to which such correlations exist in the wild remains unclear. To address this question, this thesis analyses the importance of inbreeding, measured using a panel of nine microsatellites, and variation at the MHC class II exon 2 region (DQB2) on mate choice, offspring survival and pathogen susceptibility in two wild mammal species, the grey seal (*Halichoerus grypus*) and the European wild boar (*Sus scrofa*).

The grey seal component is based on a long-term data set for two grey seal breeding colonies, the Isle of May and North Rona, and is the first to document the effect of the DQB2 region on mating strategy in seals. There are considerable inter-population differences in the extent to which the DQB region affects mating strategy. On the Isle of May levels of DQB2-dissimilarity between female grey seals and their pups significantly increase over consecutive pupping events, suggesting that as a female ages, choice for maximally MHC-dissimilar males occurs. Females on the Isle of May also produce significantly more heterozygous than homozygous offspring and have lower levels of DQB2 diversity than pups. This suggests that females are capable of choosing males that will increase heterozygosity and/or diversity in their offspring. In contrast, on North Rona there were no clear trends with any of the measures of DQB2 variation.

Comparison of the levels of inbreeding in dead and live pre-weaned grey seal pups from three island populations show that dead pups are significantly less heterozygous than live pups and that particular microsatellite alleles contribute to this significantly more than others. DQB2 diversity and heterozygosity are also significantly greater in live pups than dead pups on all islands. Furthermore, particular DQB2 alleles increase an individual's susceptibility to infections, and these alleles are significantly more prevalent in dead than live pups.

Using a detailed parasite database that was not available for grey seals, I compared variation at the DQB2 region with parasite susceptibility in the European wild boar. No significant effect of the DQB2 region on susceptibility to infection with parasites was found in this species. However, individuals infected with multiple parasites have significantly lower heterozygosity, and only carry three of the five DQB2 alleles. This finding suggests that DQB2 variation may be more important in determining parasite load rather than whether an individual is infected with parasites. There were also surprisingly low levels of DQB2 diversity and heterozygosity in this species, suggesting that genetic variation could be an important consideration in the future management of this species.

Results from this thesis suggests that variation at the MHC may be important in determining mating strategy, offspring survival and parasite diversity. However, analysis of other MHC genes, and in particular the MHC class I region in conjunction with larger sample sizes than could be achieved here may help to refine some of the patterns observed in this thesis. Furthermore, the incorporation of behavioural observations on mating behaviour, an investigation of the effect of DQB2 variation and inbreeding on post-weaning survival and the study of other grey seal populations with different demographies may prove fruitful.

Abbreviations

Table 1 A list of abbreviations used in this thesis. SI units are used throughout this thesis, except for those detailed below.

Abbreviation	
ATP	adenosine 5′- triphosphate
ANOVA	analysis of variance
Bp	nucleotide base pairs
Ci	curies (1Ci = 3.7 x 10^{10}Bq)
CTP	cytidine 5′- triphosphate
df	degrees of freedom
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DMSO	dimethylsulphoxide
DQB2	MHC class II DQB exon 2
EDTA	ethylenediamine tertaacetic acid
GLM	generalized linear model
GTP	quanosine 5′- triphosphate
HCl	hydrochloric acid
KCl	potassium chloride
M	molar
m	milli-
MHC	major histocompatibility complex
$MgCl_2$	magnesium chloride
Mol	moles
n	nano
NaAC	sodium acetate
NaCl	sodium chloride
P	probability
p	pico-
PCR	polymerase chain reaction
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SE	standard error of the mean
TE	tris-EDTA
TMAC	tetramethyl ammonium chloride
Tris	2-amino-2-(hydroxymethyl) propane1:3 diol
TTP	thyamidine 5′- triphosphate
µ	micro-

CHAPTER 1

Introduction

CHAPTER 1

Introduction

1.1 Introduction

The factors that influence mating strategy, determine offspring survival and susceptibility to pathogens in mammals involve three main components: the environment an individual lives in, behaviour amongst individuals and genetics. Of these, genetic effects are increasingly being shown to be particularly important. Genetic effects can be general and involve genome-wide measures of heterozygosity such as levels of inbreeding. Offspring born from matings between close relatives often display reduced fitness, a phenomenon known as inbreeding depression (Crnokrak & Roff 1999; Hedrick & Kalinowski 2000). Inbreeding depression is characterised by increased levels of homozygosity, which can lead to the expression of deleterious recessive alleles and/or loss of heterozygous advantage (Charlesworth & Charlesworth 1987). This is particularly evident in populations which have recently been reduced in size (Shields 1993). Alternatively, genetic effects can be more specific and involve a single locus or particular regions of the genome such as the major histocompatibility complex (MHC). The main function of the MHC is in immune response (Klein & O'Huigin 1994). More recently, genes of the MHC have also been shown to affect mate choice (Potts et al. 1991; Ober et al. 1997), kin recognition (Manning et al. 1992) and susceptibility to pathogens (Hedrick 2002).

In this chapter I first review the literature on the MHC and inbreeding. I then discuss my two study species, the grey seal (*Halichoerus grypus*) and the European wild boar (*Sus scrofa*). For the grey seal I discuss its biology and ecology, current molecular research on breeding and social structure and the main causes of mortality in the

species. I then discuss the ecology, reproduction and current status of wild boar in Spain.

1.2 The major histocompatibility complex (MHC)

1.2.1 Structure and function

Vertebrates have evolved a number of immunological defences against pathogens. Immunological responses involve a superfamily of molecules acting together and include the immunoglobulin (Ig) molecules, the T cell receptors (TcR) and the MHC which is termed the human leukocyte antigen (HLA) in humans (Dyer & Middleton 1993; Klein & O'Huigin 1994). Of these it is the MHC which acts first when challenged with a pathogen. The vertebrate MHC is a cluster of genes which includes two major subfamilies, class I and class II (Hughes & Yeager 1998). Both class I and class II MHC molecules are located on the surface of cells. Class I molecules are found on almost all types of cells and present antigens to cytotoxic T lymphocytes which continuously survey cell surfaces, and kill cells harbouring metabolically active microorganisms (Klein & O'Huigin 1994). HLA class I molecules are encoded by loci that include HLA-A, HLA-B and HLA-C (Figure 1.1).

On the other hand, class II molecules appear only on immune response cells such as macrophages, B-cells and other cells that present antigens to T cells (Knapp 2002). MHC class II molecules, unlike class I molecules, present peptides that have been digested from external sources and are encoded by the loci HLA-DP, -DQ and –DR (see Figure 1.1) (Hughes et al. 1994; Apple & Erlich 1996). The class II genes are composed of five exons which encode the α- and β-chains of the DR, DQ and the DP antigens, but nearly all polymorphism found in these genes is concentrated on the

second exon (Apple & Erlich 1996). Individuals inherit both paternal and maternal clusters of alleles or haplotypes, which are codominantly expressed (Knapp 2002).

The primary function of the MHC molecules is to bind peptide fragments within a region termed the 'peptide binding region' (PBR), and then display them on the surface of the cell (Hughes & Yeager 1998). Peptides presented within the PBR can then be recognised by the immune system as 'self' or 'non-self' (Brown & Eklund 1994). The PBR region of the class I molecule is closed, so that only peptides of a particular length (eight to ten amino acid residues) fit into this region and are bound by the termini (Klein & O'Huigin 1994). The groove of the class II molecule on the other hand is open, allowing peptides that extend beyond the PBR to be accommodated (a length range of 12 to 24 residues) (Klein & O'Huigin 1994). Once presented, the peptides are bound by antigen-specific T cell receptors of cytotoxic T cells which kill virus infected cells, or by helper T cells that stimulate T cell proliferation (Hughes & Yeager 1998).

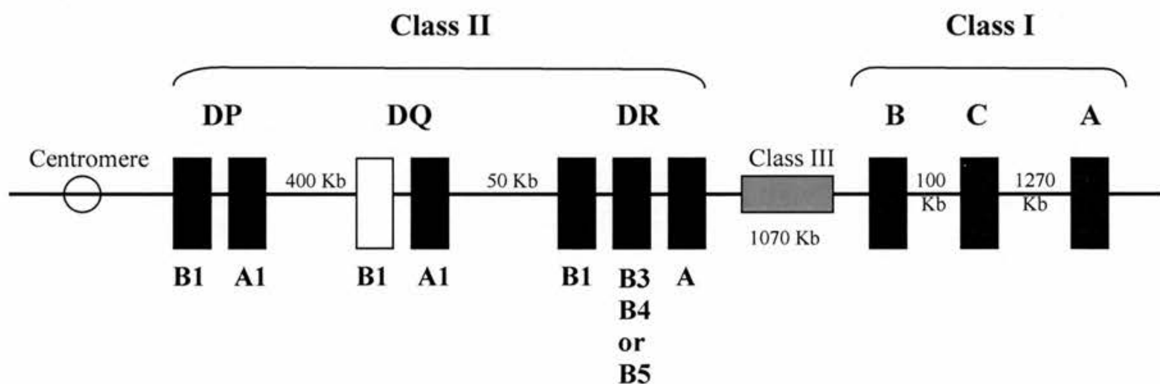

Figure 1.1 Map of the HLA complex indicating the position of the class I and II antigens (adapted from Apple & Erlich 1996). B1,3,4 and 5 indicate the β-chains and A and A1 indicate the α-chains. The region examined in this thesis is the second exon of the class II DQB region indicated as a white box.

In the past, studies investigating the MHC have focused on the human immune system and in particular cellular rejection mechanisms responsible for the failure of skin and organ transplants (Dyer & Middleton, 1993). More recently, levels of variation at the MHC have been shown to be important in disease susceptibility, kin recognition, mate choice and offspring survival in numerous species of vertebrates (for relevant literature see Table 1.1). Another characteristic of the MHC region is that MHC haplotypes can be detected by individuals olfactorily through odour cues in urine (Pause et al. 1999; Ehman & Scott 2001). It is believed that the MHC influences the odour of an individual through the production of soluble proteins that bind volatile molecules, and by influencing bacterial gut flora (van der Walt et al. 2001). This ability provides a direct mechanism through which individuals can assess different MHC haplotypes when choosing mates or associating with kin.

Table 1.1 Evidence for and against MHC-correlated mate choice, kin association and disease/parasite susceptibility in a number of species. + = positive association, – = negative association.

Species	Trait	Association	Reference
		Mate choice	
Three-spined stickleback (*Gasterosteus aculeatus*)	Dissimilarity	+	(Reusch et al. 2001)
House mouse (*Mus musculus*)	MHC dissimilarity	+	(Roberts & Gosling 2003)
House mouse (*Mus musculus*)	Mating preference	+	(Yamazaki et al. 1976; Yamazaki et al. 1978; Beauchamp et al. 1988; Yamazaki et al. 1988; Egid & Brown 1989; Potts et al. 1991; Eklund 1997a; Penn & Potts 1998)
House mouse (*Mus musculus*)	Mating preference	–	(Eklund et al. 1991; Manning et al. 1992; Eklund 1997b)
	Mate preference for non parasitised mice	+	(Kavaliers & Colwell 1995a; 1995b)
Soay Sheep (*Ovis aries*)	Mating preference	–	(Paterson & Pemberton 1997)
Human (*Homo sapiens*)	Mating preference	+	(Ober et al. 1997)
	Mating preference	–	(Hedrick & Black 1997)
Pigtailed macaque (*Macaca nemestrina*)	Pregnancy wastage	+	(Knapp et al. 1996)
Rhesus macaque (*Macaca mulatta*)	More offspring	+	(Sauermann et al. 2001)
		Kin recognition	
Arctic charr (*Salvelinus alpinus*)	Scented water	+	(Olsen et al. 1998)
House mouse (*Mus musculus*)	Nesting partners	+	(Manning et al. 1992)
Human (*Homo sapiens*)	Odour	+	(Porter & Moore 1981)
		Disease/parasite susceptibility	
Three-spined stickleback (*Gasterosteus aculeatus*)	Parasite diversity	+	(Wegner et al. 2003)
Chinook salmon (*Oncorhynchus tsawytscha*)	Haematopoietic necrosis virus (IHNV)	+	(Arkush et al. 2002)
Gila topminnow (*Poeciliopis o. occidentalis*)	Fluke (*Gyrodactylus turnbulli*) infection	–	(Hedrick et al. 2001a)
Atlantic salmon (*Salmo salar*)	Bacterial infection (*Aeromonas salmonicida*)	+	(Lohm et al. 2002)
House mouse (*Mus musculus*)	Infection with *Salmonella*	+	(Penn et al. 2002; McClelland et al. 2003)
	Malarial parasites	+	(Cigel et al. 2003)
Rhesus macaque (*Macaca mulatta*)	SIV infection	+	(Evans et al. 1999a)
Cattle (*Bos taurus*)	Bovine dermatophilosis	+	(Maillard et al. 2002)
Human (*Homo sapiens*)	Insulin-dependent diabetes	+	(Horn et al. 1988)
	HIV progression	+	(Carrington et al. 1999)
	Malaria resistance	+	(Hill et al. 1991)

1.2.2 Maintenance of MHC polymorphism

Genes of the major histocompatibility complex are the most diverse genes known to science. The origin and maintenance of the high level of diversity at the MHC is unresolved. However, sexual selection and frequency-dependent selection by pathogens are thought to be the main contributory factors. Sexual selection can either involve mating preferences for particular MHC haplotypes (Yamazaki et al. 1976) or by the selective abortion of foetuses in couples which share particular MHC alleles (Knapp et al. 1996).

Although sexual selection can determine levels of MHC polymorphism, it is the ongoing defence against pathogens that is postulated to be the main driving force behind the maintenance of such high polymorphism at the MHC (Howard 1993; Potts & Wakeland 1993; Brown & Eklund 1994). Two models have been proposed (Potts & Wakeland 1993; Hughes & Yeager 1998). Firstly, the 'overdominance' hypothesis suggests that heterozygotes are able to bind twice as many foreign peptides as homozygotes (Potts & Wakeland 1993; Hughes & Yeager 1998). This is a consequence of MHC function where different allelic products bind different arrays of peptides. Hence, it is suggested that in populations exposed to a wide array of pathogens, individuals which are heterozygous will be at an advantage because they will be able to present a broader range of antigens, and in turn resist a wider array of pathogens (Potts & Wakeland 1993; Hughes & Yeager 1998). For example, Penn et al. (2002) and McClelland et al. (2003) both report that MHC heterozygous mice have greater survival rates than homozygous individuals against infection with four different *Salmonella spp*. Similarly, disease progression and survival in humans infected with malaria and HIV has also been shown to be strongly influenced by MHC heterozygosity and the presence of particular alleles (Hill et al. 1991; Hill & Kaufman 1997).

Secondly, the 'rare allele' or 'dominance' hypothesis suggests that individuals carrying rare or particular alleles will be at an advantage due to an increased ability to fight new pathogens or those that confer protection from particular pathogens (Potts & Wakeland 1993; Hughes & Yeager 1998; Jeffery & Bangham 2000). This is because pathogen evolution will be directed against common host MHC genotypes. Both these mechanisms select for MHC diversity and both favour the evolution of mating preferences for MHC-dissimilar or heterozygous mates. These mating preferences function by preferentially producing disease resistant offspring – MHC heterozygotes, rare MHC genotypes and genotypes that change from generation to generation thereby creating a moving target for pathogen adaptation.

1.2.3 Mate choice

If MHC heterozygosity confers a selective advantage to individuals by decreasing their susceptibility to pathogens, then reproductive strategies that allow individuals to produce disease-resistant offspring can be expected to operate. To date, studies investigating the role of the MHC in mate choice have mainly been conducted on mice (Egid & Brown 1989; Eklund et al. 1991; Potts et al. 1991; Eklund 1997b) and humans (Wedekind et al. 1995; Ober et al. 1997). The majority of studies on mice have focused on inbred strains which differ only at the MHC (Manning et al. 1992; Ehman & Scott 2001). Results from these studies have indicated that MHC-dependent mate choice does occur and that homozygotes display a stronger preference for different haplotypes than heterozygotes.

In general, studies in humans support the notion that MHC diversity is maintained at least to some extent by assortative mating. For example, one of the first studies investigating the relationship between HLA haplotypes and mate choice was the

'T-shirt' study (Wedekind et al. 1995). The T-shirt study involved 49 women who were asked to rate the attractiveness of the odour of T-shirts worn by three MHC-similar and three MHC-dissimilar men (Wedekind et al. 1995). Results from this study suggest that women generally preferred the odour of MHC-dissimilar men and that this pattern was reversed in women taking oral contraceptives (Wedekind et al. 1995). However, this study has been criticised because it provides indirect evidence for mate preference (Penn & Potts 1999).

Evidence for MHC-correlated mate choice in humans has also been documented in a population called the Hutterites (Ober et al. 1997). The Hutterites are a small, isolated North American community established by a founder population of approximately 400 individuals in the 1870s (Ober et al. 1997). The group is noted for their large sibships, communal lifestyle and limited number of five-locus HLA haplotypes (HLA-A, -B, -C, -DR, and DQ) (Ober et al. 1997). Studies investigating HLA haplotype-similarity between couples in the Hutterite population have revealed that individuals will avoid a potential mate who has a haplotype that is similar to their own (Ober et al. 1997). Despite positive associations between the MHC and mate choice, a study by Hedrick & Black (1997) provides counter-evidence for MHC-correlated mate choice. Hedrick & Black's (1997) study on a population of South American Indians found no evidence that MHC homozygous deficiency was due to dissortative mating. However, it has since been suggested that their research was inadequate for detecting MHC-correlated mating preference because the sample size was too small and the study did not consider extra-pair copulations (Penn & Potts 1999).

Although the majority of studies on humans and laboratory mice have revealed a positive relationship between MHC-dissimilarity and mate choice, only a handful of

studies have investigated MHC-dependent mate choice in natural populations (for examples see Paterson & Pemberton 1997; Ekblom et al. 2004). Furthermore, the comparative difficulty in publishing negative results makes it hard to assess the extent to which the MHC influences mate choice. The only published study to have reported no association between the MHC and mate choice was conducted on Soay sheep (*Ovis aries*) by Paterson & Pemberton (1997). More studies on natural populations and a higher rate of publication of negative results are required to provide a clearer indication of the effects of the MHC on mate choice.

1.2.4 Kin Recognition

The ability of animals to discriminate between relatives and non-relatives plays a crucial role in social interactions and has been explained in the context of Hamilton's (1964) theory of inclusive fitness. Individuals are expected to behave more altruistically towards kin who are genetically similar to themselves, thereby indirectly increasing their own fitness (West et al. 2002). The MHC has been suggested as an important component of kin recognition because it is used in the immune system as a means to discriminate between self and non-self (Brown & Eklund 1994). In addition, because the MHC has been linked to odour production, individual body odour can be used as a possible means by which individuals can associate with individuals with whom they share a similar MHC haplotype (Pause et al. 1999; Ehman & Scott 2001). For example, a laboratory study on juvenile Arctic charr (*Salmo salar*) revealed that when fish had the choice between water scented by an MHC-identical sibling and a sibling with a different MHC genotype, they preferred the former (Olsen et al. 1998). Similarly, studies on house mice (*Mus musculus*) have shown individuals to prefer the

odour of kin (Ehman & Scott 2001) and that females prefer communal nesting partners that share particular alleles of the MHC (Manning et al. 1992).

1.2.5 The MHC in pinnipeds

Despite numerous studies providing positive support for MHC-correlated patterns of mate choice, fitness or kin association in terrestrial mammals (see Table 1.1), no study to date has investigated whether or not this trend extends to aquatic mammals. Unlike studies of terrestrial mammals, MHC studies in pinniped populations have revealed low levels of allelic diversity (Trowsdale et al. 1989; Slade 1992; Hoelzel et al. 1999; Lento et al. 2003). Slade (1992) found only five alleles at the class II MHC DQB exon 2 locus in the southern elephant seal (*Mirounga leonina*) using restriction length polymorphism, compared to 25 in humans (Apple & Erlich 1996). Similarly, the number of alleles at exon 2 of the class II MHC DQB locus for two congeneric phocid seal species, the southern and the northern (*Mirounga angustirostris)* elephant seal, and two congeneric otarid species, the Antarctic (*Arctocephalus gazella*) and New Zealand (*Arctocephalus fosteri*) fur seals ranged between only two to eight alleles (Hoelzel et al. 1999). Lento et al. (2003) also found only two alleles at the DQB gene in the New Zealand sea lion (*Phocarctos hookeri*) using single strand conformation polymorphism.

The low levels of allelic diversity of the MHC found in pinnipeds have been suggested to be the result of low levels of exposure to pathogenic selection compared to terrestrial mammal species (Slade 1992). Slade et al. (1992) have theorised that reduced exposure to pathogens may in turn explain why marine mammals may be susceptible to occasional mass mortalities such as the distemper events in the European North Sea in 1988 and 2003, when a novel pathogen entered the population killing up to 17,000 harbour seals (*Phoca vitulina*). However, all these species have at some point

been extensively hunted and reduced variability at the MHC may in fact be due to a previous population bottleneck. Hence, the exact cause of low levels of MHC diversity in marine mammals has yet to be determined.

1.3 Inbreeding depression

Inbreeding depression, like the MHC region, can be an important determinant of fitness (Charlesworth & Charlesworth 1987) and is well documented by laboratory studies (Wright 1977; Festing 1979) and in domestic animals (Prichner 1985). However, few studies have investigated the relationship between fitness and inbreeding in natural populations (for examples see Margulis 1998; Hoglund et al. 2001; Emery & McCauley 2002; Acevedo-Whitehouse 2003). The primary reason for this is the need for detailed pedigree information in order to determine levels of relatedness between given individuals. In natural populations of long lived organisms, there are inherent difficulties in measuring fitness traits (Hutchings & Ferguson 1992) and few studies yield sufficiently detailed pedigree information (Pemberton et al. 1992; Keller et al. 2001). More recently, molecular markers such as microsatellites have been utilised to study inbreeding in natural populations by directly calculating measures of parental similarity, thereby bypassing the need for pedigree data.

Microsatellites or simple sequence repeats are tandomly repeated motifs of two to six bases found in all eukaryotic genomes studied to date (Tautz & Renz 1984; Litt & Luty 1989; Tautz 1989; Weber & Wong 1993). They are present in coding and non-coding regions and display a high degree of length polymorphism (Tautz 1989). Length variation in microsatellites appears to be the result of mutations due to slippage during DNA replication (Weber & Wong, 1993). The abundance and variability of microsatellites throughout the genome has resulted in their widespread use in

population studies (Queller & Goodnight 1989; Queller et al. 1993). This is because allele frequencies can be easily calculated and individuals can unequivocally be designated as a homozygote or heterozygote. In addition, the use of the polymerase chain reaction (PCR) to amplify microsatellites has enabled small amounts of DNA of poor quality to be amplified to single nucleotide resolution. The relatively small size of microsatellite loci, with most less than 200 base pairs (bp) in length, means that PCR product can then be resolved on standard polyacrylamide gels (Tautz & Renz 1984; Litt & Luty 1989; Tautz 1989; Weber & Wong 1993). Alleles can thus be reliably scored for large numbers of individuals over numerous loci making it possible to study large populations.

Genetic methods for estimating levels of inbreeding using microsatellites have moved from the use of simple heterozygosity to three alternate measures which are considered to be more sensitive (see Coulson et al. 1998; Slate et al. 2000; Amos et al. 2001a). First, mean d^2 is based on evolutionary distances between microsatellite alleles (Coulson et al. 1998). Mean d^2 takes into account the stepwise mutation process of microsatellites that leads to new alleles, usually increasing or decreasing by one repeat unit (Weber & Wong 1993). Inbreeding values are calculated as the squared difference in length between alleles at a locus averaged across all loci used (Coulson et al. 1998). Second, standardised heterozygosity (SH) calculates heterozygosity but the score at each locus is weighted by the average heterozygosity at that locus (Coltman et al. 1999). This method avoids a potential bias associated with individuals not being genotyped at all loci, which frequently occurs in molecular studies. Third, internal relatedness (IR) is based on allele sharing, where the frequency of every allele counts towards the final inbreeding value (Amos et al. 2001a). This method weights shared rare alleles more heavily than shared common alleles and is a direct derivative of Queller & Goodnight's

(1989) 'relatedness' value (Amos et al. 2001a). Hence, IR provides an estimate of the relatedness between an individual's parents.

1.4 Study species

This thesis investigates the relationship between levels of inbreeding and variation at the DQB2 region on mating strategy, offspring survival and pathogen susceptibility in two wild mammal species. The first species, the grey seal was considered an ideal species in which to investigate the relationship between levels of inbreeding and variation at the DQB2 region on mating strategy and offspring survival for two reasons. First, mating strategies known to operate in grey seal colonies can lead to incestuous matings. Second, studies on two of the populations of grey seal included in this thesis, the Isle of May and North Rona, have spanned 18 years and there is a good long-term database available. The second species, the European wild boar was considered a good model species in which the effect of the DQB2 region on parasite susceptibility could be investigated because of the availability of an excellent detailed parasite dataset that was not available for the grey seal. Detailed information on the biology and ecology of both species are provided below.

1.4.1 The grey seal, *Halichoerus grypus* (Fabricius 1791)

1.4.1.1 Classification, distribution and movement

The grey seal, *Halichoerus grypus*, belongs to the group Pinnipedia which contains three extant families: the Otariidae (sea lions and fur seals), the Phocidae (true seals) and the Odobenidae (walrus). The grey seal is the only member of its genus (Bonner 1999). The name *grypus*, which translates to 'hook nosed', was given due to the 'Roman nose' profile of adult males (Bonner 1999). Grey seals are sexually dimorphic.

Males reach 1.95 to 2.3 meters in length and weigh between 230 to 310 kg (Bonner 1999). Females are considerably smaller, reaching 1.65 to 1.95 meters in length, and weighing between 105 to 186 kg (Bonner 1999). Females have a lighter, more uniform background fur colour than males with dark patches or spots around the neck (Figure 1.2) (Bonner 1981). In contrast, males are uniformly dark grey or brown with only a few pale patches (Figure 1.2) (Bonner 1981). Maximum ages of grey seals have been documented at 46 years for females and 44 years for males (Bonner 1981). Most females become pregnant for the first time at the age of four or five years and give birth to a single pup in the following year after a gestation period of approximately ten months (Bonner 1981).

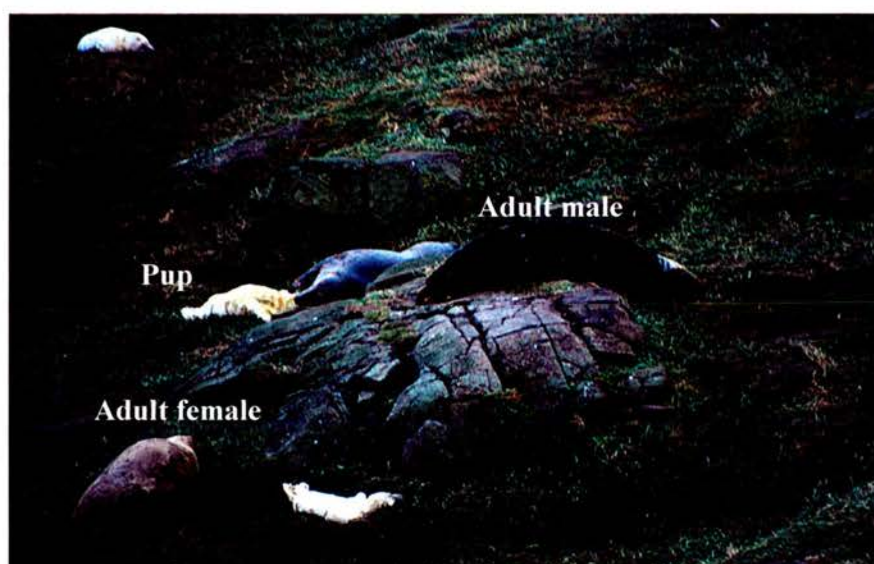

Figure 1.2 An adult male grey seal surrounded by adult females and their pups. Males have darker fur and are larger than females.

The grey seal occurs in three distinct regions: the north-west Atlantic, the Baltic and the north-east Atlantic (Figure 1.3) (Davies 1957). These three populations are believed to be reproductively isolated with no mixing since the last interglacial period (Davies 1957). Although grey seals occur over a large geographic area, approximately half of the world's population breed on offshore islands around the British Isles, in particular the north and west of Scotland (Davies 1957; Boyd & Campbell 1971; Bonner 1972). The main breeding locations of grey seal populations in Scotland are in the Hebrides, the Orkneys, North Rona and the Isle of May (Figure 1.4) (Boyd & Campbell 1971; Allen 1995). Previous molecular studies on the Isle of May (located off the east coast of Scotland) and North Rona (north-west Scotland) have revealed little or no mixing even between these populations, indicating fine scale population differentiation, high levels of site fidelity of adults and philopatry of pups (Allen et al. 1995; Allen 1995).

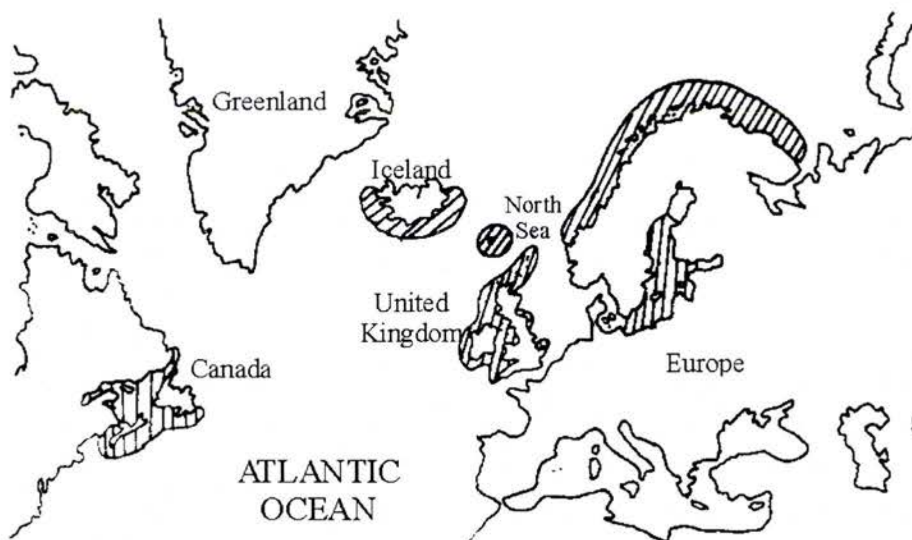

Figure 1.3 Worldwide distribution of the grey seal (adapted from Bonner 1981).

Figure 1.4 Map of the United Kingdom indicating the main breeding islands of grey seals (adapted from Allen 1995).

The study of grey seal movements outside the breeding season has recently been made possible by the use of radio (Thompson et al. 1991) and satellite (McConnell et al. 1992) tagging, providing direct measurements of the movement of free-ranging grey seals. For example, McConnell et al. (1992) and Hammond et al. (1993) used transmitters to relay information via satellite, reducing the need to follow seals by boat and allowing information to be obtained immediately and over several months. From these studies it has been shown that there is high individual variability in the distances moved and that most grey seals move large distances, up to 264 km in some cases, outside the breeding season (McConnell et al. 1992). Hence, it appears that grey seals form social groups predominantly during the breeding season but are widely dispersed at most other times of the year.

1.4.1.2 Breeding behaviour and spatial organisation

Grey seal social organisation and breeding behaviour has been described in several studies on colonies throughout their range (Anderson et al. 1975; Boness & James 1979). However, most studies have focused on populations of grey seals breeding on Sable Island, Nova Scotia (Boness & James 1979; McCulloch & Boness 2000) and North Rona, Scotland (Boyd et al. 1962; Boyd & Campbell 1971; Anderson et al. 1975; Pomeroy et al. 1994; Twiss et al. 1994).

In Britain, grey seals aggregate in colonies at the beginning of autumn (Boyd & Cambell 1971). During the breeding season, adult males arrive at the colony after female groups have become established (Bonner 1981). Behavioural studies of grey seals have suggested that the degree of aggression by dominant males toward neighbouring males during the breeding season is an indication of reproductive success, with a small number of dominant males gaining a disproportionate number of

paternities (Bonner 1981; Twiss et al. 1998). This behaviour is characteristic of a polygynous mating strategy (Bonner 1981).

The recent introduction of genetic techniques for assessing paternity has, however, revealed an alternate strategy. For example, Amos et al. (1993) could not assign paternity to the local dominant male in 36 % of cases, leading them to suggest that aquatic mating may play a larger role than previously thought. Further work by Amos et al. (1995) revealed large numbers of full siblings within the colony although the dominant males fathered disproportionately few. Here it was suggested that many full siblings result from choice by females favouring previous parental combinations, such that polygyny and partner fidelity may be operating simultaneously (Amos et al. 1995). Not only did mate choice seem to be occurring in grey seals, maternal half-siblings who have different fathers were significantly more diverse than expected from random mating, leading Amos et al. (2001b) to suggest that females may be selecting for genetically diverse partners.

The presence of alternative mating strategies in grey seals may be advantageous because selection of maximally dissimilar mates will be favoured in order to reduce the effects of inbreeding in closed polygynous systems (Amos et al. 2001b). It has been suggested that because seals are long-lived animals with high site fidelity, different strategies to maximise genetic mixing would offset the potential for increased levels of inbreeding caused by polygynous mating (Amos et al. 2001b). Although a number of strategies appear to be occurring, the mechanism and selective advantages of such alternate strategies are still unknown.

In addition to patterns of breeding behaviour, the spatial distribution of females in relation to levels of relatedness has also been investigated to determine the degree of site fidelity and possibility of kin association. Twiss et al. (1994) and Pomeroy et al.

(1994; 2000a; 2000b) showed that male and female grey seals display high site fidelity and that females and their daughters are found pupping together in subsequent years even in areas other than the daughters' natal site. It was suggested that movement of females and offspring may be driven by kin recognition (Pomeroy et al. 2000a; 2000b), which may in turn result in fine scale population structuring within the colony (Pomeroy et al. 1994).

Further studies have since investigated the ways in which topographical features affect the distribution of individuals, and how relatedness varies spatially (Pomeroy et al. 2000a; Twiss et al. 2000). Pomeroy et al. (2000a) found that seals from the centre of the colony were more closely related than to all other seals than the colony average. These locations were also areas of high pupping success and were the first areas occupied at the beginning the breeding season (Pomeroy et al. 2000a). Although patterns so far have suggested that kin recognition may be occurring, no studies to date have conclusively shown kin association in grey seals. Hence, although the mating strategies and spatial relationships in grey seal colonies are slowly being resolved, the mechanisms driving these strategies and patterns still remain unclear.

1.4.1.3 Causes of grey seal mortality

Studies on the causes of death in wild grey seals have been ongoing since 1979 (for examples of early work see Anderson et al. 1979; Baker 1980; Baker et al. 1980). In particular, studies by Baker over the last 20 years have provided detailed descriptions on the incidence of mortality in pups, juveniles and adult grey seals (Baker 1980; 1984; 1987; 1988; 1989; Baker et al. 1980; 1998; Baker & Baker 1988; Baker & Martin 1992). In the majority of cases, the primary cause of death in grey seal pups is either starvation, peritonitis, trauma, gastroenteritis, drowning or still birth, although there are

numerous other, rarer causes (Baker et al. 1980; Baker 1984). The primary causes of death in juveniles and adult grey seals have been identified as drowning in fishing gear, starvation (mainly in juveniles) and respiratory diseases (Baker 1987).

Grey seals have also been susceptible to outbreaks of parapox ('sealpox') and calicivirus, with the most detailed recorded incidence in England occurring in Cornwall in 1991/92 (Simpson et al. 1994). Furthermore, although grey seals are susceptible to morbilivirus (phocine distemper) they do not seem to be as severely affected as harbour seals, which were affected by an epidemic estimated to have killed 17,000 seals in 1988 (Simpson et al. 1994). A second, but slightly more severe, epidemic occurred in European harbour seals in 2002. Unpublished data from this epidemic showed that grey seals were infected but did not succumb to the disease (see http://www.smru.st-and.ac.uk /CurrentResearch.htm/scos.htm).

The causes of mortality in grey seal pups have also been investigated in relation to colony density (Twiss et al. 2003). Twiss et al. (2003) used fine scale maps of daily seal locations in a geographic information system (GIS) database to determine female density at a range of spatial scales around each pup on the date of death. However, they failed to find any relationship between density of breeding females and pup mortality, although there were fewer mortalities in prime pupping habitats. Whether this is the result of habitat quality, interactions between individuals, quality of mothers and/or maternal care is unknown.

1.4.2 The European wild boar, *Sus scrofa* (Linnaeus 1758)

1.4.2.1 Distribution and reproductive biology

The wild boar, *Sus scrofa* (Linnaeus 1758) (Figure 1.5) belongs to the family Suidae (Order Artiodactyla) and is native throughout Europe, Asia and North Africa (Clutton-Brock 1999; Rajkovic-Janje et al. 2002; Vernesi et al. 2003; Wilson 2003). More recently, wild boar have been introduced into Southern Africa, Australia, New Zealand, the United States of America, central and south America, and many oceanic islands such as the West Indies and the Galapagos islands (Vernesi et al. 2003). Populations of wild boar in Europe have become considerably fragmented in recent years due to changes in land use such as increased intensity of agricultural practices and loss of woodland habitats (Gortazar et al. 2000). Despite this, *Sus* populations appear to be increasing and expanding throughout Europe.

Male wild boars are usually solitary, but have been known to aggregate in groups of up to 50 individuals, mainly at watering holes and during the breeding season (Vaughan et al. 2000). Females form matriarchal groups of related females which occasionally contain immature males (Clutton-Brock 1999). Reproduction is seasonal and females give birth to up to ten offspring at a time which are usually fathered by a dominant male (Clutton-Brock 1999). The age of puberty for sows ranges from 8 to 24 months and pregnancy lasts 115 days with a sow giving birth to four to six piglets on average (Nahlik & Sandor 2003). Following birth, piglets will remain close to their mother however after four to five days will also suckle from other lactating females within the group (Nahlik & Sandor 2003). After 12 to 16 weeks piglets are weaned and usually remain within the group until they reach puberty (Nahlik & Sandor 2003).

Figure 1.5 An adult male wild boar.

1.4.2.2 Wild boar in Spain

The European wild boar is the most widely distributed ungulate in the Spanish mainland and its range and density has increased considerably over the last two decades (Gortazar et al. 2000). Wild boar is also Spain's most important large game species and as such has been extensively managed by high wire fencing, artificial feeding and restocking with farm bred individuals (Gortazar et al. 2000). As a result of intensive management some wild boar hunting estates resemble extensive breeding facilities where there is almost no sanitary care (Vicente pers.com). These changes in wildlife management have given rise to concerns regarding the control and spread of infectious diseases (Gortazar et al. 2002; 2003; Vicente et al. 2002).

Like commercially bred species of swine, wild populations of *Sus scrofa* are susceptible to a wide range of infectious diseases and parasites (de-la-Muela et al. 2001; Gortazar et al. 2002; 2003; Rajkovic-Janje et al. 2002; Ellis et al. 2003). Wild boar have also been implicated as a possible reservoir for bovine tuberculosis (Gortazar et al. 2003) and Aujeszky's disease (pseudorabies) (Gortazar et al. 2002). Furthermore, there is considerable risk of transmission of helminths to wild and commercial suids during translocations of wild boar (Fernandez-de-Mera et al. 2003a; 2003b). Since this species is prevalent throughout Europe and is an important game species, the impact and spread of infectious diseases and parasites and the factors contributing to susceptibility are of particular interest. In particular, diseases such as brucellosis and tuberculosis are not only an important disease in swine, but also have public health implications because they are important zoonoses in humans (MacMillan 1999).

1.5 Thesis Aims

This thesis examines the way in which the MHC class II DQB exon 2 region (DQB2) affects mating strategy and offspring survival in the grey seal, and how this interacts with levels of inbreeding. I also examine the importance of the DQB2 region on parasite susceptibility in the European wild boar, using a database that would have been difficult to compile for grey seals. In summary, the main aims of this thesis are:

1. To isolate and characterise MHC class II DQB exon 2 (DQB2) alleles for the grey seal and the European wild boar.
2. To optimise the use of denaturing gradient gel electrophoresis (DGGE) to genotype grey seal and European wild boar individuals for DQB2 alleles.

3. To use variability at the DQB2 region and inbreeding measured using nine microsatellites to address the following questions regarding grey seal breeding behaviour and social organisation:
    - Does DQB2 variation affect mating strategy in the grey seal?
    - Do levels of inbreeding and variation at the DQB2 region influence pre-weaning pup survival?
    - Is there variability in the effect of the DQB2 region and levels of inbreeding on pup survival between island populations, between different causes of mortality, and does this vary with pup age?
4. To determine if the DQB2 region affects parasite susceptibility in the European wild boar.

1.6 Data analysis

All statistical analyses were conducted using R statistical program version 1.6.2 (Ihaka & Gentleman 1996) and SPSS 11. All parametric statistical tests data were tested for normality using Levene's test of equality of error variances (Sokal & Rohlf 1995). Data which deviated from normality were transformed (see specific examples in each chapter for tests used) (Sokal & Rohlf 1995). When this failed to successfully stabilise variances, an equivalent non-parametric test was used (see specific examples throughout). Additional data analyses are described in detail in each chapter.

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CHAPTER 2

General materials and methods

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2.1 Introduction

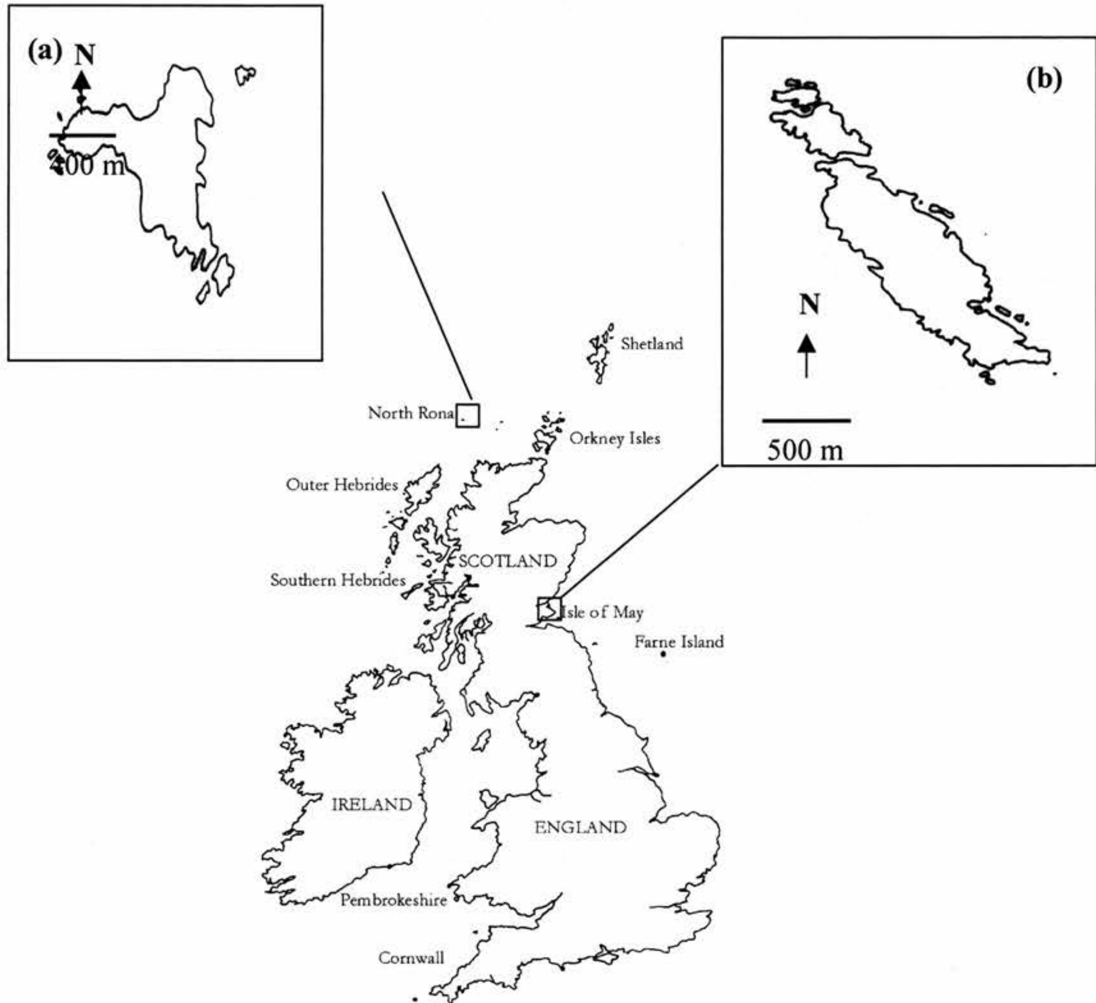
This chapter describes the study sites and the methods used to genotype grey seal individuals for nine microsatellite loci. I also provide the results of tests for Hardy-Weinberg equilibrium, the presence of null alleles and linkage disequilibrium. Detailed descriptions of methods specific to subsequent chapters are not included here.

2.2 Study sites

2.2.1 Isle of May

The Isle of May is a small island approximately 2 km long and 0.5 km wide, located in the Firth of Forth 9.5 km off the coast of Fife (56°11'N; 2°33'W) (Figure 2.1). It is a relatively newly founded colony (around 1975) and has increased in numbers from approximately 30 to 1770 individuals over the last 25 years (Harwood & Wylie 1987; Pomeroy et al. 1999). It is now the biggest single island colony of breeding grey seals on the east coast of the UK. On the Isle of May, the grey seal breeding season commences in early October and ends mid-to late December. Seals breeding on the Isle of May have been the subject of ongoing behavioural, physiological and molecular studies by the Sea Mammal Research Unit (SMRU; http://www.smru.st-and.ac.uk) based at St Andrews University since the 1980s.

Figure 2.1 Map of the United Kingdom indicating the two UK study sites **(a)** North Rona and **(b)** the Isle of May (adapted from (Allen 1995).



2.2.2 North Rona

North Rona is a small rocky island with a total area of approximately 120 ha, located 75.5 km NNW off Cape Wrath, Scotland (59° 06′ N, 05° 50′ W) (Figure 2.1) (Boyd & Campbell 1971). North Rona pup production has remained relatively stable at around 1200 to 1500 individuals for about a decade (Pomeroy et al. 1994; Pomeroy et al. 1999). The breeding season commences in late September and ends in late November. Grey seals breeding on North Rona have also been the subject of intensive long-term studies conducted by SMRU. The current research began in 1985.

2.2.3 Sable Island

Sable Island is a crescent-shaped, partially vegetated sand bar approximately 35 km long and up to 1.5 km wide, located 288 km ESE off Halifax, Nova Scotia, Canada (43° 55′ N, 60° 00′ W) (Figure 2.2). The island supports the largest grey seal breeding colony in the world, with a pup production of 25,000 in 1997 (Bowen et al. 1999). The breeding season on Sable Island starts at the end of December and ends around mid to late February (Boness & James 1979).

2.3 Grey seal sample preservation

Blood samples collected by previous researchers were stored in an 2:1 ethylenediamine tetraacetic acid (EDTA) coated vacutainer. Tissue samples collected prior to 2002 were preserved in 20 % dimethyl sulphoxide (DMSO) saturated with sodium chloride (Amos & Hoelzel 1991) and stored at –20 °C. Skin samples collected in 2002 and 2003 were preserved and stored in 95 % ethanol at room temperature. Full details on samples collected and the number of samples successfully genotyped for microsatellites and DQB2 are listed in Table 2.1.

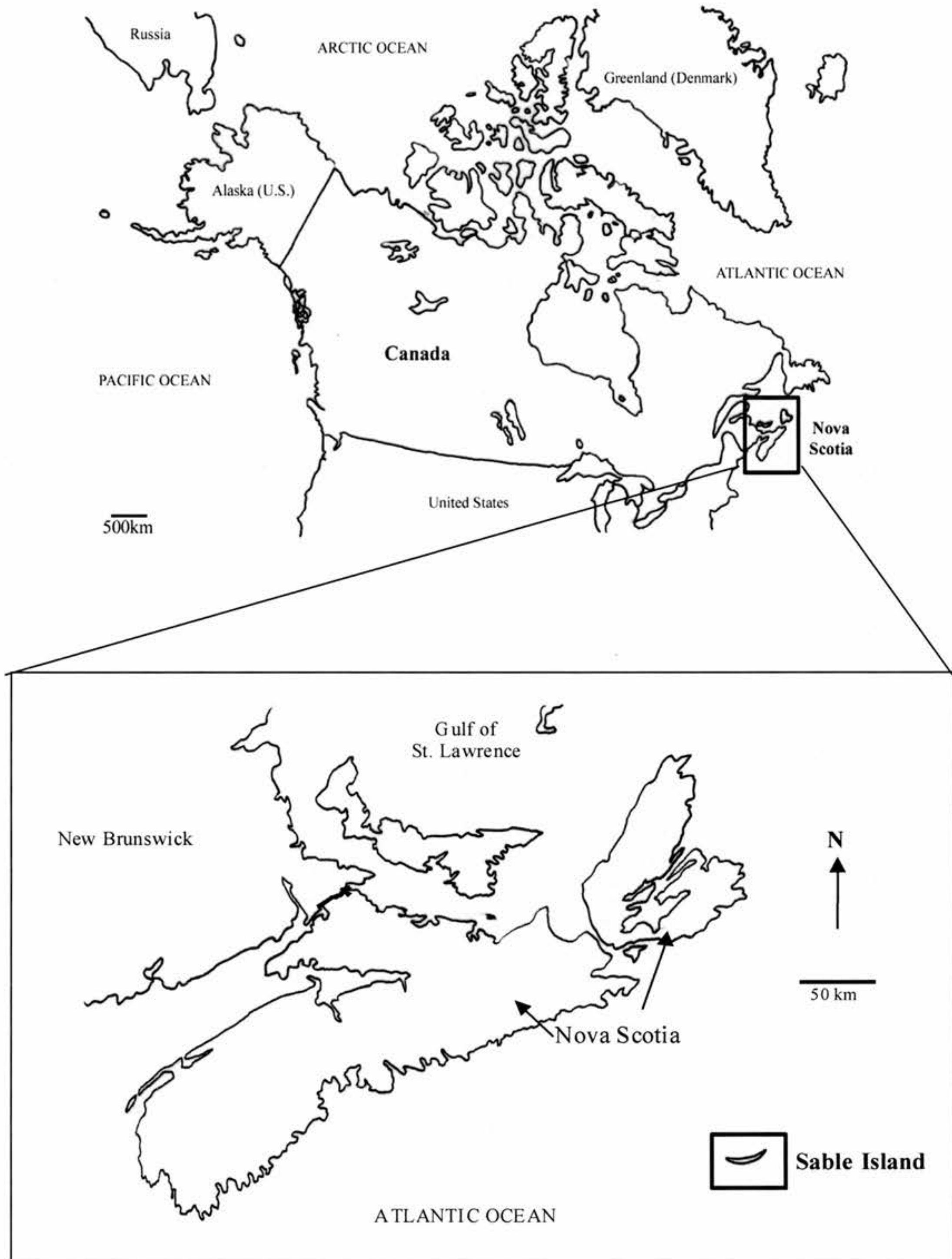


Figure 2.2 **(a)** Map of Canada indicating the position of Nova Scotia and **(b)** the location of the Sable Island grey seal breeding colony off the east coast of Nova Scotia.

Table 2.1 Details of the samples available, samples which required re-extraction and total number of samples which have been successfully genotyped for microsatellites and the DQB2 region per island. NA = Not available.

Island	Sample	Skin sample (n)	Blood sample (n)	Number of samples re-extracted	Microsatellite genotyped (n)	DQB2 genotyped (n)
North Rona	Dead pups	87	0	0	77	30
	Live pups	130	110	92	122	104
	Adult females	0	60	16	27	27
Isle of May	Dead pups	411	0	209	252	253
	Live pups	161	54	70	98	110
	Adult females	20	29	5	28	28
Sable Island	Dead pups	40	NA	0	40	39
	Live pups	28	NA	0	28	28

2.4 Microsatellite genotyping

2.4.1 Extraction of genomic DNA from skin samples

This study builds on an existing database for grey seals in which the first samples were collected in 1986 on North Rona and 1991 on the Isle of May. In this study, total genomic DNA was extracted from samples taken from 239 live pups, 538 dead pups and 21 adult females. Total genomic DNA was extracted from skin samples following standard phenol-chloroform extraction procedures (Sambrook et al. 1989).

Each skin sample was cut in half (approximate size 2 x 2 mm) and placed in a 1.5 ml eppendorf containing 330 μl of digestion solution; 100 mM NaCl, 50 mM Tris pH 8.0, 20 mM EDTA, 1 % SDS, 500 μg of proteinase K and 250 μg RNAse A. Samples were placed in a 55 °C oven for two hours and then on a rotating wheel overnight at 37 °C. 300 μl of a 1:1 phenol:chloroform mix was added to each extraction and placed on a rotating wheel for 30 minutes and then centrifuged at 13,000 rpm for ten minutes. Approximately 200 μl of supernatant was then removed and placed in a clean 1.5 ml tube. An equal volume of chloroform was added, the tube vortexed briefly and centrifuged at 13,000 rpm for ten minutes. The supernatant (approximately 200 μl) was pipetted off and placed in a clean 1.5 ml tube. DNA was then precipitated out by adding 100 μl of 8M ammonium acetate and 500 μl of 95 % ethanol and placed at –70 °C for 30 minutes. Tubes were then centrifuged at 13,000 rpm for 15 minutes and the solution removed leaving the DNA pellet as dry as possible. Each pellet was washed by adding cold 70 % ethanol, inverting the tube a few times and centrifuging at 13,000 rpm for five minutes. Ethanol was then pipetted off and the pellet dried for approximately ten minutes at 40 °C. DNA was resuspended in 200 μl of 1 X TE (10 mM Tris, 1 mM EDTA), and stored at -20 °C.

The quality and quantity of DNA was determined by electrophoresis of 10 μl of DNA solution with 5 μl of loading buffer on a 0.7 % agarose gel. DNA was electrophoresed at 60 V for 50 minutes, stained with ethidium bromide and DNA visualised using UV light at a wavelength of 320 nm. This extraction procedure yielded approximately 20 μg/ml of DNA from skin samples.

This study also includes DNA extracted by previous researchers from samples collected from 1986 to 1999. Blood and skin samples collected prior to 1994 were phenol-chloroform extracted (n = 253). Some samples collected from 1994 to 1999 were extracted by previous researchers using the same digestion protocol but with the addition of 5 % Chelex 100® chelating resin (n = 322) following (Walsh et al. 1991). DNA samples that had been Chelex extracted had been stored in a 5 % Chelex solution containing, 100 mM NaCl, 50 mM Tris pH 8.0, 20 mM EDTA and stored at -20 °C. Due to problems with DNA quality (see chapter three section 3.2.5) all samples from which DNA had been extracted using Chelex were re-extracted using the phenol-chloroform extraction protocol as described above.

2.4.2 Microsatellite amplification

Genotyping of all grey seal samples was conducted using a panel of nine locus-specific microsatellite primers (Table 2.2). PCR amplification was conducted in a final volume of 12 μl containing; 2 μl of template DNA, 1 x Thermalase buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1 % Tween 20, 0.1 % Gelatin, 0.1 % IGEPAL), 0.1 mM dA, dG, dTTP, 0.01 mM dCTP, 0.25 units of *Taq* polymerase, 0.2 μM of each primer, 60 mM TMAC and 0.01 μCi [α^{32}P]-dCTP. Magnesium chloride and formamide concentration varied between primers (for $MgCl_2$ and formamide concentrations for each locus see Table 2.3). PCR reactions were overlaid with 10 μl mineral oil in 96 well microtitre

plates. PCR cycling parameters are detailed in Table 2.3. Each microtitre plate contained four positive standards with alleles of known size. Following PCR amplification, 10 µl of formamide stop solution (80 % formamide, 10 mM EDTA, 1 mg/ml xylene cyanol, and 1 mg/ml bromophenol blue) was added.

PCR products were separated using gel electrophoresis. Prior to loading PCR product on an acrylamide gel, the product was denatured for four minutes at 96 °C. For each individual, 4 µl of denatured product was loaded into a single well of a 96-well comb. Alleles were separated on a 6 % polyacrylamide gel (made from pre-mixed 'Sequagel' solutions, National Diagnostics) run at a constant temperature of 45 °C at 70 watts for between one and two and a half hours depending on the size of the amplified product. Following electrophoresis, gels were vacuum dried at 80 °C for two hours and exposed to X ray film (Kodak MXB film) for between 4 to 24 hours at –70 °C depending on the level of radioactive incorporation.

Table 2.2 Loci and primer sequences for PCR amplification of grey seal microsatellites. F = Forward, R = Reverse.

Locus	Primer sequence (5′-3′)	Number of alleles	Product size
Hg3.6	F: AGATCACATTCTTTTTATGGCTG R: GATTGGATAAAGAAGATGTGAGGG	8	85-101
Hg4.2	F: AATCGAAATGCTGAGCCTCC R: TGATTTGACTTCCCTTCCCTG	8	139-165
Hg6.1	F: TGCACCAGAGCCTAAGCAGACTG R: CCACCAGCCAGTTCACCCAG	6	150-166
Hg6.3	F: CAGGGGACCTGAGTGCTTATG R: GACCCAGCATCAGAACTCAAG	6	219-229
Hg8.9	F: TGTTAACTATCTGGCACAGAGTAAG R: TTTCCTATGGGTTCTACTCTC	11	197-217
Hg8.10	F: AATTCTGAAGCAGCCCAAG R: GAATTCTTTTCTAGCATAGGTTG	10	183-201
Hgd.2	F: ACCTGCCATAGTGCTCATC R: GAGCCAACTAAGACAAGCC	8	111-141
SGPv9	F: TAGTGTTTGGAAATGAGTTGGCA R: ACTGATCCTTGTGAATCCCAGC	7	160-172
SGPv11	F: GTGCTGGTGAATTAGCCCATTATAAG R: CAGAGTAAGCACCCAAGGAGCAG	8	160-174

Table 2.3 PCR amplification cycling parameters for all microsatellite loci. Primer annealing temperature (A and B) varies between loci and are indicated below.

Step	Process	Temperature (°C)	Time (Seconds)	Number of cycles
1	Denaturation	72	120	1
2	Denaturation	94	30	7 = Hg4.2, Hg6.3, Hgdii
	Primer annealing	**A**	30	6 = all other loci
	Extension	72	30	
3	Denaturation	94	15	25
	Primer annealing	**B**	30	
	Extension	72	30	
4	Extension	72	120	1

Primer annealing temperatures (°C), $MgCl_2$ and formamide concentration per locus

Primer	A	B	$MgCl_2$ (mM)	Formamide (%)
Hg3.6	56	58	2.2	0.27
Hg4.2	54	58	3.4	0.5
SGPv9	50	52	2.5	0.5
SGPv11	58	60	2.5	0.5
Hg6.1	58	60	1.5	0
Hg8.1	56	58	2.5	0.5
Hg8.9	54	56	2.5	0.2
Hgdii	56	60	2.45	0.5
Hg6.3	54	58	2.9	0.5

2.4.3 Microsatellite scoring

Each amplified microsatellite produced a distinctive, locus-specific set of bands (for examples see Figure 2.3). Each allele was identified as a strong band which usually had a series of fainter stutter bands either above or below at two bp intervals (Figure 2.3). These stutter bands are thought to be due to slippage events during the PCR reaction (Hauge & Litt 1993) and did not interfere with genotype scoring.

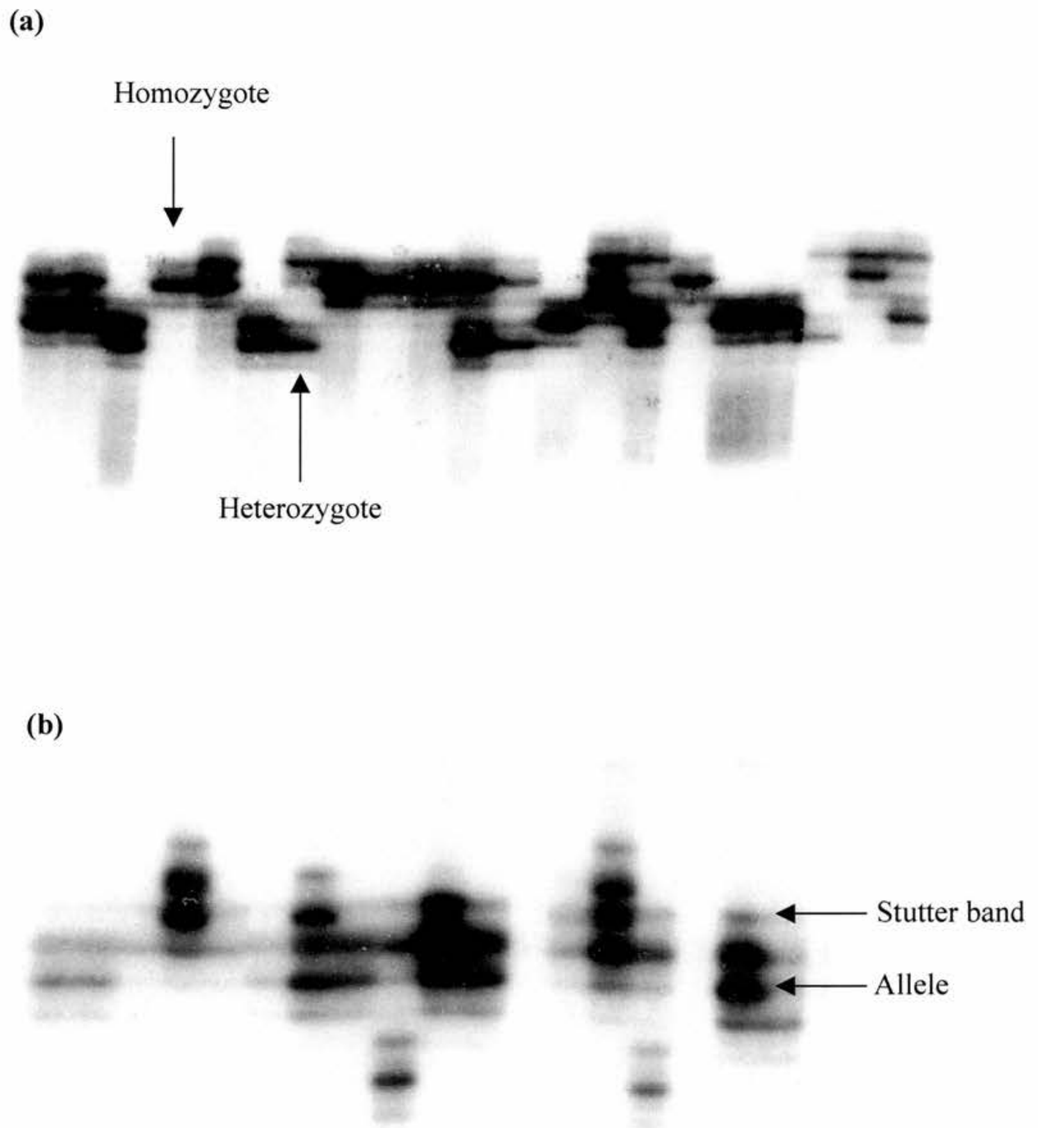


Figure 2.3 Samples amplified for microsatellite locus SGPv9 **(a)** and Hg3.6 **(b)**. In figure **(a)** a homozygous and heterozygous individual are indicated. In figure **(b)** the difference between a stutter band and an allele is indicated.

2.4.4 Estimates of inbreeding

As outlined in chapter one, studies investigating levels of inbreeding in wild populations do not usually have access to detailed pedigree information from which levels of relatedness can be estimated. More recently, the use of microsatellites has allowed the relatedness of two individuals to be estimated without the need for pedigree information. In this thesis I use microsatellite generated data to calculate the level of inbreeding in grey seal pups with comparison between live and dead pups within the pre-weaning period. This was estimated using two measures, internal relatedness (IR) and standardised mean d^2 (S mean d^2). In all cases, inbreeding calculations were performed using 'IRmacro', an Excel© macro (Microsoft Office 2000) written in Visual Basic by W. Amos. For each pup, IR was calculated as;

$$\text{Internal relatedness} = \frac{(2H - \sum f_i)}{(2N - \sum f_i)} \qquad (2.1)$$

where H is the number of loci that are homozygous, N is the number of loci and f_i is the frequency of the ith allele contained in the genotype (Amos et al. 2001). When calculated across multiple loci individuals born to 'unrelated' parents usually have IR values that are approximately normally distributed and more or less centred around zero. Negative IR values suggest relatively 'outbred' individuals and high positive values are suggestive of relatively inbred individuals.

The second measure, mean d^2 was calculated as;

$$\text{mean } d^2 = \sum_{i=1}^{n} \frac{(i_a - i_b)^2}{n} \qquad (2.2)$$

where i_a and i_b refer to the length of each allele at each locus averaged over n loci (Coulson et al. 1998). This measure was then standardised by dividing each value by the maximum observed at that locus to calculate standardised mean d^2 (Hedrick et al. 2001).

$$\text{Standardised mean } d^2 = \frac{1}{n}\sum_{i=1}^{n}\frac{(i_a - i_b)^2}{(i_a - i_b)^2_{\max}} \tag{2.3}$$

This reduces the influence of highly polymorphic loci on the overall measure by ensuring that each locus contributes equally to the final score.

In addition to calculating inbreeding levels in live and dead pups using IR and S mean d^2 I also calculated standardised heterozygosity (SH). This measure is commonly used as a measure of inbreeding in studies on wild populations and is the simplest multilocus measure, where the proportion of homozygotes versus heterozygotes at each locus is calculated and then weighted by the expected heterozygosity at that locus. However, a strong relationship between IR and SH is known to exist (Amos et al. 2001). In order to determine if IR was correlated with SH using the grey seal dataset, I investigated the relationship between IR and SH. Comparison of IR and standardised heterozygosity revealed that these two measures are highly correlated in the grey seal (Pearson correlation value = -0.979, n = 250, $P < 0.001$). Of the two, IR is considered to be more informative because it weights allele sharing by the frequency of those alleles (Amos et al. 2001). As such only IR and S mean d^2 were used as an estimate of inbreeding for this thesis.

2.5 Microsatellite characteristics

2.5.1 Allele frequencies and Hardy-Weinberg equilibrium

Although the loci used in this study have been tested for Hardy-Weinberg equilibrium (HWE), the presence of null alleles and linkage disequilibrium by (Allen 1995), I re-tested the same loci using samples collected by me and other researchers in order to ensure that the same holds true for this dataset. Allele frequencies were determined by direct counting from all dead and all live animals from each island population across all loci. Allele frequency distributions at each locus for pups on each island are shown in Figure 2.4. Observed and expected heterozygosities were calculated using GENEPOP v. 3.3 (Raymond & Rousset 1995). Genotypes of live and dead pups at each locus from each island were tested for deviation from HWE using the exact test option in GENEPOP. HWE states that the expected heterozygosity in a large random mating population with no selection, mutation, or migration should be constant from generation to generation (Hardy 1908).

Observed heterozygosity (H_0) was calculated as:

$$H_0 = \sum \frac{p_{ij}}{n} \tag{2.4}$$

and expected heterozygosity (H_E) as:

$$H_E = 1 - \frac{(n\sum p_i^2 - 1)}{n-1} \tag{2.5}$$

Where, p_i and p_j are the frequency of the ith and jth allele, p_{ij} is the number of heterozygotes for alleles i and j, and n is the number of individuals (Nei & Roychoudhury 1974). The significance of deviation from HWE was calculated using a Markov chain following Guo and Thompson (1992).

All nine loci were found to be highly polymorphic, with heterozygosity ranging from 0.564 to 0.839 (Table 2.4). There were six alleles at the least variable locus and eleven alleles at the most variable. Five loci were found to deviate significantly from HWE for dead pups on North Rona and the Isle of May (Hg. 3.6, Hg4.2, SGPv9, Hg8.1 and Hgdii) (Table 2.4). While it is difficult to discriminate between the presence of null alleles and the possibly of inbreeding in dead pups, tests for HWE in live pups reveal no deviation from HWE suggesting that deviation from HWE in dead pups is potentially due to inbreeding. Hence, despite five of the loci showing signs of disequilibrium for dead pups all loci were used in the final data set for further analysis.

Table 2.4 Tests of HWE for live and dead pups samples from three islands. Observed heterozygosities (H_O) and significance values for deviations from HWE for each microsatellite locus in live and dead pups on each island are depicted.

	North Rona				**Isle of May**				**Sable Island**			
	Live		Dead		Live		Dead		Live		Dead	
Locus	H_O	*P*	H_O	*P*	H_O	*P*	H_O	*P*	H_O	*P*	H_O	*P*
Hg3.6	0.613	0.528	0.766	**0.000**	0.815	0.756	0.714	**0.007**	0.821	0.823	0.744	0.418
Hg4.2	0.564	0.056	0.720	0.704	0.774	0.999	0.664	**0.002**	0.786	0.573	0.725	0.789
SGPv9	0.839	0.858	0.720	**0.002**	0.792	0.817	0.765	**0.007**	0.643	0.443	0.692	0.753
SGPv11	0.722	0.595	0.716	0.922	0.651	0.594	0.656	0.174	0.643	0.648	0.650	0.702
Hg6.1	0.624	0.135	0.680	0.451	0.633	0.737	0.672	0.127	0.667	0.178	0.718	0.273
Hg8.1	0.810	0.948	0.743	0.066	0.823	0.845	0.732	**0.000**	0.615	0.208	0.750	0.725
Hg8.9	0.787	0.054	0.729	0.062	0.837	0.950	0.801	0.761	0.741	0.302	0.692	0.141
Hgdii	0.672	0.295	0.620	0.069	0.739	0.494	0.612	**0.005**	0.607	0.115	0.650	0.149
Hg6.3	0.788	0.385	0.693	0.247	0.702	0.260	0.635	0.079	0.821	0.952	0.700	0.062
Mean	0.713		0.710		0.752		0.694		0.705		0.702	

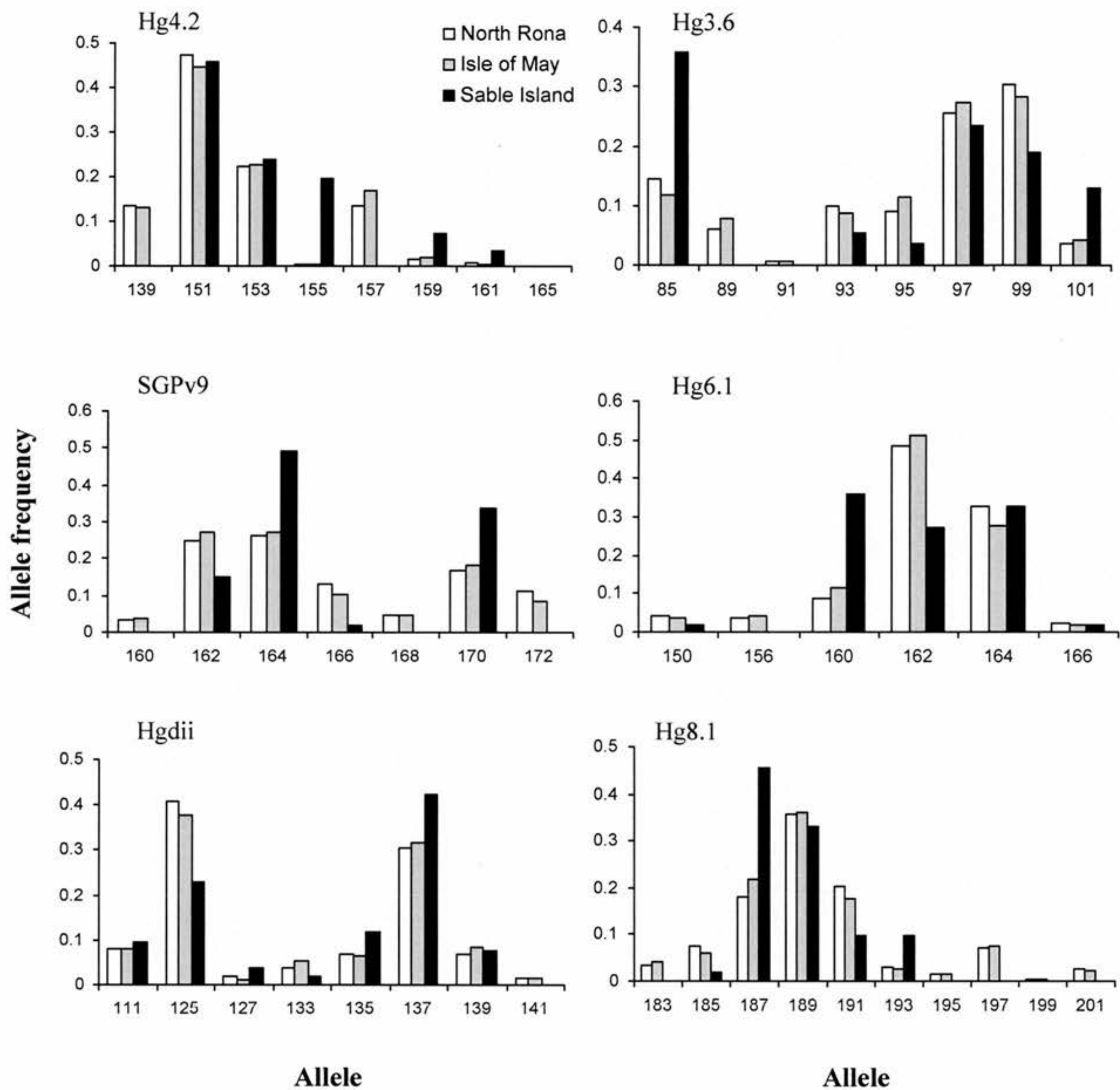


Figure 2.4 Grey seal allele frequency distributions for each of the nine microsatellites from three different island populations; North Rona, the Isle of May and Sable Island.

Figure continues overleaf.

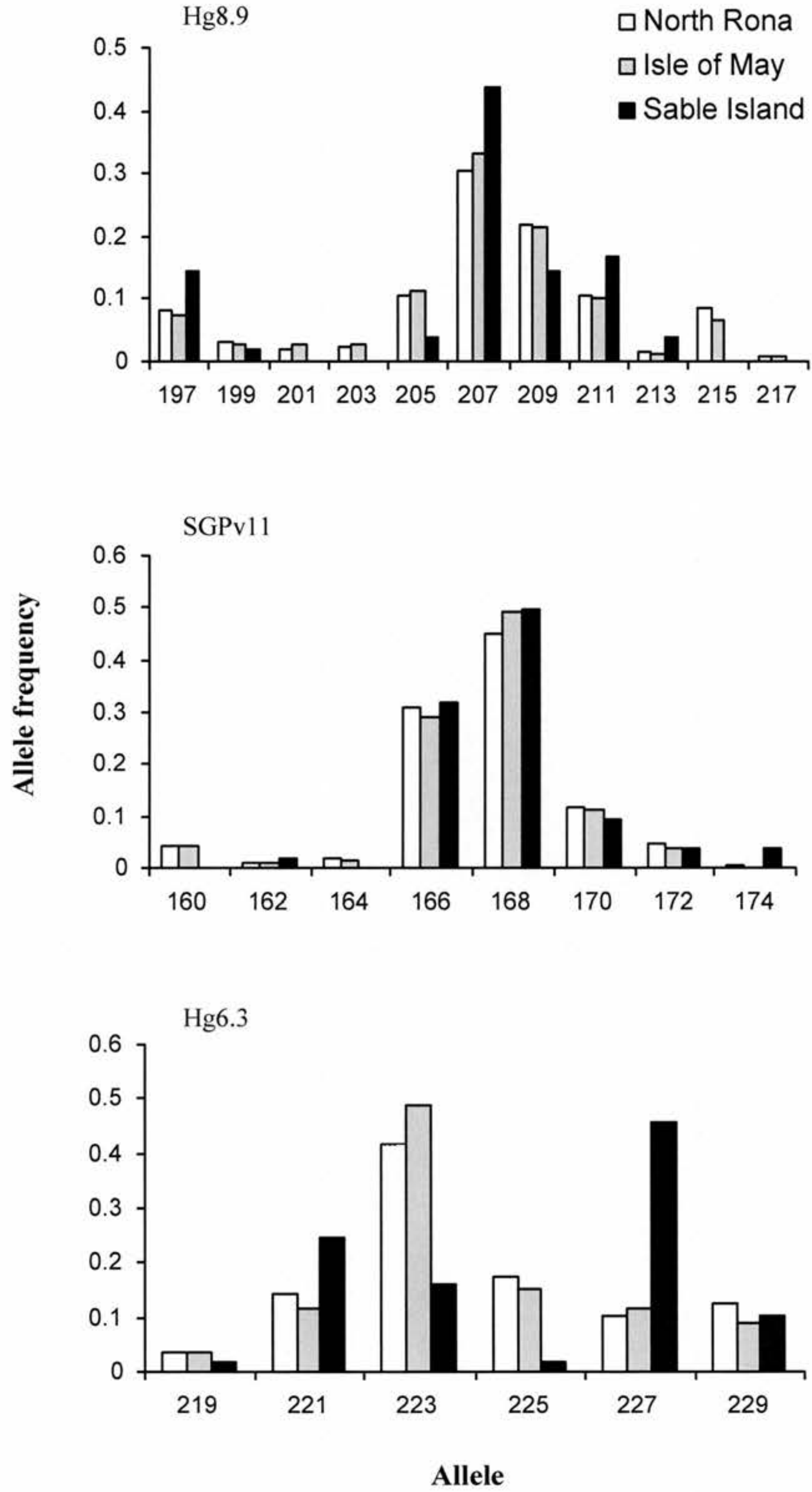


Figure 2.4 Continued.

2.5.2 Null alleles

When microsatellite loci fail to yield a visible amplification product this is termed a null allele or non-amplifying allele (Pemberton et al. 1995). Null alleles probably arise through a point mutation in one or both primer binding sites or deletion of the locus (Callen et al. 1993). At high frequencies null alleles can result in some individuals not amplifying at all and null homozygotes (Pemberton et al. 1995). The presence of null homozygotes can also lead to deviations from HWE (Pemberton et al. 1995). Null allele frequencies were calculated using GENEPOP. Fewer than 5 % of the alleles at given locus were null.

2.5.3 Linkage disequilibrium

If alleles from two loci do not associate independently in a population the two loci are said to be in linkage disequilibrium (Hartl & Clark 1997). Linkage disequilibrium can occur if loci are physically linked (i.e. are located close together) or if they are null alleles (Hartl & Clark 1997). Linkage disequilibrium can also arise because of the presence of close family members, population structure or founder effects. To test for associations between loci, the exact, unbiased probability of disequilibrium was estimated using the genotypic disequilibrium option in GENEPOP. There was no evidence of linkage disequilibrium between any of the pairs of loci.

2.5.4 Estimation of population divergence

Since the grey seals examined in this thesis came from three distinct island populations, I determined genetic differentiation amongst these islands by comparing microsatellite allele frequencies between islands. (Allen 1995; Allen et al. 1995) previously reported that North Rona and the Isle of May are genetically distinct. To confirm the results

obtained by (Allen et al. 1995) and provide an indication of the degree of differentiation with Sable Island, I conducted the same analysis but included additional samples for the UK populations and Sable Island.

The degree of population subdivision between islands was estimated using Wright's fixation index (F_{ST}), which measures the reduction in heterozygosity of a subpopulation due to random genetic drift (Wright 1965). F_{ST} values were calculated for live pups using GENEPOP and were tested for significance using the method described by (Workman & Niswander 1970). F_{ST} and the corresponding χ^2 test are shown in Table 2.5. Eight of the nine loci show highly significant F_{ST} values with the only non-significant locus being SGPv11 for all comparisons made (Table 2.5). Overall F_{ST} values were highly significant for each of the island comparisons indicating population subdivision (Table 2.5).

Table 2.5 F_{ST} values with corresponding χ^2 values of significance for each microsatellite locus for live grey seal pups. Numbers of degrees of freedom are displayed in parentheses after each χ^2 value. *** = $P < 0.001$; * = $P < 0.05$.

Locus	**North Rona vs Isle of May**		**North Rona vs Sable Island**		**Sable Island vs Isle of May**	
	F_{ST}	χ^2	F_{ST}	χ^2	F_{ST}	χ^2
Hg3.6	0.0075	36.5(7)***	0.0352	74.4(7)***	0.0546	106.3(7)***
Hg4.2	0.0124	68.9(7)***	0.0481	116.2(7)***	0.0582	113.3(7)***
SGPv9	0.0160	66.7(6)***	0.0757	137.2(6)***	0.0603	100.6(6)***
SGPv11	0.0021	10.2(7)	0.0050	10.57(7)	-0.0044	8.6(7)
Hg6.1	0.0268	93.1(5)***	0.3451	521.1(5)***	0.3440	478.2(5)***
Hg8.1	0.0128	80.1(9)***	0.2550	693.1(9)***	0.2568	642.5(9)***
Hg8.9	0.0026	19.9(10)*	0.1886	626.5(10)***	0.2095	582.41(10)***
Hgdii	0.0074	36.0(7)***	0.2792	590.2(7)***	0.2390	465.1(7)***
Hg6.3	0.0221	76.8(6)***	0.1082	163.4(6)***	0.1457	243(6)***
Overall	0.0120	66.7(8)***	0.1606	388.0(8)***	0.1636	363.8(8)***

2.6 Error checking

Microsatellite genotyping can be prone to error through PCR amplification and genotyping (Kobayashi et al. 1999; Bonin et al. 2004). In order to ensure that genotypes for each individual were correct, 96 samples were randomly selected and genotyped twice for each locus. All gels were blind scored twice to estimate levels of error associated with scoring. Overall error rate for genotyping and scoring was 0.9 % and 2.01 % respectively which was considered acceptable for use of these data in further analysis.

2.7 Conclusion

This chapter describes the materials and methods used to genotype grey seal samples for nine microsatellite loci, and the tests of microsatellite characteristics. All loci were highly polymorphic, and for live pups all were in HWE. Deviation from HWE for five loci in dead pups was considered to be a result of inbreeding due to homozygous excess, rather than a high frequency of null alleles. Furthermore, there was no evidence of linkage disequilibrium and the frequency of null alleles was sufficiently low as to have a negligible effect on subsequent analysis. Therefore, the genotypes obtained from the nine microsatellites used here are considered appropriate for determination of levels of inbreeding in grey seals. In addition, F_{ST} values for all three populations indicate that there is significant subdivision between the three grey seal colonies.

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CHAPTER 3

Identification and characterisation of MHC alleles

CHAPTER 3

Identification and characterisation of MHC alleles

3.1 Introduction

The MHC has become an increasingly popular region of the genome to investigate because of its functional importance in the immune system as well as the role it may play in mate choice, kin recognition and offspring survival (Tregenza & Wedell 2000). Initially, serological typing methods were used to discriminate between MHC alleles (Horn et al. 1988). However, a single serological antigen can be encoded by numerous alleles. Not only does this method fail to identify the full compliment of MHC alleles carried by an individual, it is also very time consuming (Gaur et al. 1989). More recently, molecular methods such as single strand conformation polymorphism (SSCP) (Glavac & Dean 1993), restriction fragment length polymorphism (RFLP) (Fischer & Lerman 1979), temperature gradient gel electrophoresis (TGGE) (Wartell et al. 1990), denaturing gradient gel electrophoresis (DGGE) (Fischer & Lerman 1983; Knapp et al. 1997b) and direct sequencing (Knapp et al. 1997b) have been used to investigate allele variation at the MHC. Of these techniques, DGGE is considered to be one of the best methods to discriminate variation at the MHC (Knapp et al. 1997b). This technique is very sensitive and all single-base substitutions, frameshifts, and deletions less than about 10 bp can be resolved (Cariello & Skopek 1993).

3.1.1 Denaturing gradient gel electrophoresis

DGGE is an electrophoretic process that can identify single base changes in a segment of DNA (Fischer & Lerman 1983). Small fragments, usually 200 to 700 bp in length are run on an acrylamide gel within which is a denaturing gradient beginning at a low concentration and ending in a high concentration. The denaturing environment is produced within the acrylamide gel using urea and formamide and through a constant uniform temperature, usually between 50 to 65 °C (Knapp et al. 1997a; 1997b). Initially fragments migrate through the gel according to molecular weight, but as they progress into higher denaturing conditions the DNA strand begins to 'melt' (Fischer & Lerman 1983). DNA is said to 'melt' when the fragment which is initially double stranded becomes partially single stranded (partial melting) (Figure 3.1) (Fischer & Lerman 1983; Lerman & Silverstein 1985). Rather than partially melting in a zipper-like manner, most fragments melt in stepwise fashion or in discrete segments called 'melting domains' (Knapp et al. 1997b). The melting temperature of each domain is sequence specific and as the fragment moves through the gel it is said to 'unzip' beginning at the low melting domain, creating a branched molecule (see Figure 3.1) (Fischer & Lerman 1983). Partial melting severely retards the progress of the molecule in the gel and a mobility shift is observed. This allows point mutations in the sequence to be detected by differences in mobility as bands on an acrylamide gel (see Figure 3.1).

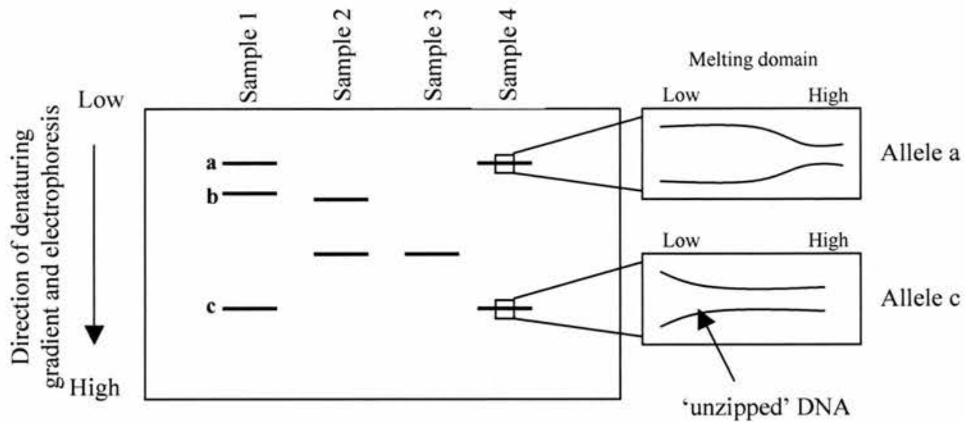

Figure 3.1 Schematic diagram of four samples that have been electrophoresed through a parallel denaturing gradient gel. The denaturant is graded from a low to high concentration in the direction of electrophoresis. Differences in the 'melting' profile of each sequence are seen as bands on an acrylamide gel (a, b, c). Allele a and b 'unzip' earlier than allele c at the lower melting domain and stop 'unzipping' depending on the composition of the sequence.

3.1.2 Aims

This chapter describes the optimisation of DGGE followed by direct sequencing of alleles to determine MHC diversity at the second exon of the class II DQB locus (DQB2) for the grey seal and European wild boar. It describes the design of primers, optimisation of PCR conditions, use of DGGE, problems associated with this technique, and methods of cloning.

3.2 Materials and methods

In order to investigate variation at the MHC, I used the second exon of the MHC class II DQB gene (DQB2). This region was chosen because it encompasses the putative peptide binding region (PBR) which is responsible for binding and presenting peptides to T cells (Hughes et al. 1994; Hughes & Yeager 1998; Hoelzel et al. 1999). Variation in this region will influence an individual's ability to bind and present peptides, directly affecting their ability to respond to certain immune challenges. The DQB2 region has also been shown to be highly polymorphic in humans (Meyer & Thomson 2001) and mice (Hughes & Yeager 1998), which is important when investigating patterns of mate choice, disease and parasite susceptibility (Boyson et al. 1996; Knapp et al. 1996; 1998; Evans et al. 1999b). This region also displays high inter-species sequence conservation between mammals, facilitating comparison between taxa as well as allowing for the design of primers.

3.2.1 Primer design

The DQB2 region of the MHC has yet to be formally identified for the grey seal and as such there are currently no published primers or sequences for this species. However, a study by Hoelzel et al. (1999) developed primers to investigate variation at the DQB2 region for four species of seal: *Arctocephalus fosteri*, *Arctocephalus gazella*, *Mirounga leonina* and *Mirounga angustirostris*. Although the primers developed by Hoelzel et al. (1999) produced a PCR product of 142 bp this does not include the entire length of the exon (Hoelzel et al. 1999). Despite the small product size, the forward primer was close to the start of the second exon. Hence, for the purpose of this study, the forward primer was taken from Hoelzel et al. (1999), while the reverse primer was developed by comparing published class II DQB exon 2 region sequences over a range of taxa. These

were obtained from GENBANK at the National Centre for Biotechnology Information webpage (http://www.ncbi.nlm.nih.gov) (see Table 3.1). Reverse primers were designed to produce a 271 bp PCR product. Each primer combination (see Table 3.1) was tested on a panel of eight grey seal DNA samples under various DNA concentrations and temperature profiles to determine which pair was to be used (see section 3.2.3 for full details).

In contrast, the entire MHC region has been extensively studied in *Sus scrofa* (Chardon et al. 1999a; Ando et al. 2001), and there are published sequences from both class I and class II regions of the MHC (Chardon et al. 1999b). I used primers described by Omi et al (1999) to amplify 305 bp of the DQB2 region that also included the PBR for *Sus scrofa* (Table 3.2).

Table 3.1 Sequence alignment of a single DQB2 sequence from a sheep (*Ovis aries)*, cow (*Bos taurus)*, pig (*Sus scrofa)*, orangutan (*Pongo pygmaeus*) and dog (*Canis familiaris*). This alignment assisted in the design of the reverse primer that was then used to amplify alleles from the DQB2 region in the grey seal. Primers are indicated in bold.

Species	Sequence 5′ – 3′
	Forward primer
Sheep	**AGG ATTTC GT GTACCAG** TT T AAG GGCCT GT GT TACTT CAC CAACGG GACG GAGC GGGTG C
Cow	------ --- ------ ---- ---- --A-- --- T---- ------ --- ------ ----
Pig	------ --- ------ ---- --TT -GA-- -C-- ---- TT ----A --- ------ ----
Orangutan	------ --- ------ ---- ---C -A--- -C-- ------ ------ --- ----C ----
Dog	------ --- ------ ---- ---- -GA-- -C-- T---- ------ --- ------ ----
	10 20 30 40 50 60
Sheep	GGCTCGT GAC CAGATA CTTC TACAA CCGGC AGGA GGAC GT GC GCTTC GAC AGCGAC TGGG
Cow	-T---- --- ------ -A-- ----- --A-G --- -T-- -C ------ --- ------ ----
Pig	-G---- -G- ---G-G GG-- ----- --A-G --- -C-- -- ------ --- ------ GT--
Orangutan	-TT---- --- ------ -A-- --T-- ---AG --- -T-- -C ------ --- ------ GT--
Dog	--TTC- --- T-A--- -A-- --T-- ----G --- -T-- -- ------ --- ------ GT--
	70 80 90 100 110 120
Sheep	GCGAGTA CCG GGCGGT GACG CCGCT GGGGC GGCG GCAA GC CG AGTAC TGG AACAGC CAGA
Cow	A------ --- ----C- ---C ----- ----- --C -GCC -- ------ --- ------ ----
Pig	-G----- --- ------ ---C ----- ----- --C -ACC -- ---C-- --- ---G-- ----
Orangutan	AG-T--- --- ------ ---- ----- ----- C--C TG-C -- ------ --- ------ ----
Dog	-G----- --- ------ CŃ-- GA--- C---- --C CTCG -- T------ --- ---CCG ----
	130 140 150 160 170 180
Sheep	AGGACAT CAT GGAGCG GGTG CGGGC CGAGG TGGA CACG GT GT GCAGA CAC AACTAC CAGG
Cow	------ -C- ------ -AC- ----- ----- --- --G- -- ------ A-- ------ ----
Pig	----G- -C- -----A -AA- ----- ----- --- ----- -- ----A- --- ------ --A
Orangutan	---AG- -C- ----A- -ACC ----- G---T --- ----- -- ------ --- ------ --T
Dog	----GA G-- ------ ---A ----- ----C --- ----- -- ------ --- ------ GG-T
	190 200 210 220 230 240
Sheep	CAGAGCT CAT CACCTC CTTG **CA GCGGC GAG GTGAGC G** **Reverse primer**
Cow	TG--AGC -CC -TT-A- --G- ----- ----- --- --
Pig	T----GA AGG ---GA- -C-- ----- ----- --- --
Orangutan	T------ -CG ---GA- -C-- ----- ----- --- --
Dog	TG--AG AGC- --- -A-G- -- ------ --- ----T -
	250 260 270 280

Table 3.2 Details of all reverse primers tested and the primer pair used in this study (indicated in bold) plus the GC clamped reverse primer (indicated in italics) for PCR amplification of the DQB2 region in the grey seal and wild boar. R = Reverse, F = Forward.

Species	Primer	5′-3′	Source
Grey seal	**DQB2**	**F: TCGTGTACCAGTTTAAGGGC**	Hoelzel et al (1999)
	DQB 1	R: CGCCGCTGCAAGATCGTTCTCT	Alignment
	DQB 3	R: GCTGCAAGATCGTTCTCT	Alignment
	DQB 4	R: CGCCGCTGCAAGATCGTTC	Alignment
	DQB 5	R: GATTTCGTGTTCAGTTTAAGGGCG	Alignment
	K2DQB	**R: CGCTCACCTCGCCGCTG**	Alignment
	DQBGC	***R:CGCCCGCCGCGCCCCGCGCCCGGCCCG CCGCCGCCCCCGCCGCTCACCTCGCCGCTG***	WinMelt®
Wild Boar	**2EF**	**F: CGGAATTCCCCGCAGAGGATTTCGTG**	Omi et al (1999)
	2EF	**R: AGGGATCCGTACCTCGCCGCTG**	Omi et al (1999)
	2EFGC	***R:CGCCCGCCGCGCCCCGCGCCCGGCCCGCCG CCGCCCCCGCAGGGATCCGTACCTCGCCGCTG***	WinMelt®

3.2.2 PCR conditions

In order to optimise PCR conditions for grey seal and wild boar samples, a series of tests were conducted on four DNA samples (for details on all optimisation parameters tested see Table 3.3). A larger sample volume (48 μl instead of 12 μl) was needed for DGGE and this could not be adequately obtained by increasing the PCR reaction volume. Amplification was either not successful or produced less product in reactions greater than 24 μl. Hence, each sample was amplified twice and pooled to produce a final volume of 48 μl. In cases where amplification was problematic, final reaction volumes were reduced to 12 μl, which in most cases improved PCR yield. Where PCR was not successful, DNA concentrations were increased from 8 ng to 16 and 20 ng per reaction. In some cases this resulted in successful PCR amplification. Most PCR

amplifications of DQB2 for the grey seal and wild boar were performed in a final volume of 24 μl under the following conditions: 8 ng DNA template, 1 x Thermalase buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1 % Tween 20, 0.1 % Gelatin, 0.1 % IGEPAL), 100 μM dTTP, dATP, dCTP, 0.25 units of *Taq* polymerase and 25 pmol of each primer.

PCR reactions were conducted in 96 well microtitre plates, overlaid with 10 μl mineral oil and with cycling parameters detailed in Table 3.4. To determine which samples were successfully amplified, 8 μl of PCR product were separated electrophoretically on a 1.5 % agarose gel, stained in 1 x TBE (0.79 M Tris, 0.79 M boric acid, 1 mM EDTA) buffer bath containing 0.5 μg/ml ethidium bromide and the product visualised under UV light at a wavelength of 320 nm. All subsequent staining of agarose gels in this chapter was conducted in this manner.

Table 3.3 The parameter and concentration, volume or amount tested during PCR optimisation for grey seal and wild boar samples.

Parameter tested	Concentration, volume or amount
	Grey seal
PCR volume	12, 24, 48, 60, 120 and180 μl
DNA concentration	40, 8, 4, 0.8, 0.4 and 0.04 ng
$MgCl_2$ concentration	0.8, 1, 1.5 and 2 mM
Primer concentration	10, 25 and 50 pM
dNTP's	0.1, 0.5 and 1 mM
Annealing temperature	45 to 68 °C
Cycle length	60 or 90 seconds
	Wild boar
DNA concentration	40, 8, 6, 4, 0.8 and 0.4 ng
Sample volume	12 and 24 μl
Annealing temperature	55 to 60 °C
Primer concentration	25 and 50 pM

Table 3.4 PCR amplification cycling parameters for DQB2 alleles in the grey seal and wild boar.

Step	Process	Temperature (°C)	Time (Seconds)	Number of Cycles
		Grey seal		
1	Denaturation	94	350	1
2	Denaturation	94	60	35
	Primer annealing	55	60	
	Extension	74	90	
3	Extension	72	600	1
		Wild boar		
1	Denaturation	94	350	1
2	Denaturation	94	60	35
	Primer annealing	55	60	
	Extension	72	60	
3	Extension	72	350	1

3.2.3 Addition of GC clamp

In order to increase the number of single base changes that can be distinguished by DGGE a GC rich region is often added to the end of either the forward or reverse primer (Sheffield et al., 1989). Addition of the GC clamp prevents the DNA fragment from denaturing completely during DGGE by creating a region which is in a higher melting domain to that of the rest of the sequence (Sheffield et al., 1989). In doing so, the sequence of interest becomes the first melting domain and the resolution of the technique is increased. WinMelt™ Version 2 (Biorad) is a computer program which calculates and graphs theoretical melting profiles. The WinMelt program calculates the midpoint temperature (Tm) at which each base pair is at 50/50 equilibrium between a helical and melted state. It then plots the Tm versus the sequence base to create a melting profile for a given sequence. This program was used to visualise the effect of adding a 40 bp GC clamp to the 5′ and 3′ end of nine *Sus scrofa* sequences to determine correct placement of the GC clamp. However, for the grey seal there are no published sequences. Therefore, a melt profile was produced using published sequences from the multiple species alignment used in the design of the reverse primer (see Table 3.1). Inspection of WinMelt graphs showed that the addition of a GC clamp to the 5′ end of *Sus* DQB2 sequences and the five sequences from Table 3.1 produced a melting profile with the GC clamp in a higher melting domain to the rest of the sequence (see Figure 3.2 and 3.3). Hence, all subsequent PCR reactions for the amplification of the DQB2 region using DNA from grey seal and wild boar samples were conducted using the reverse primer as indicated in Table 3.2 but with the addition of a GC clamp at the 5′ end of the primer.

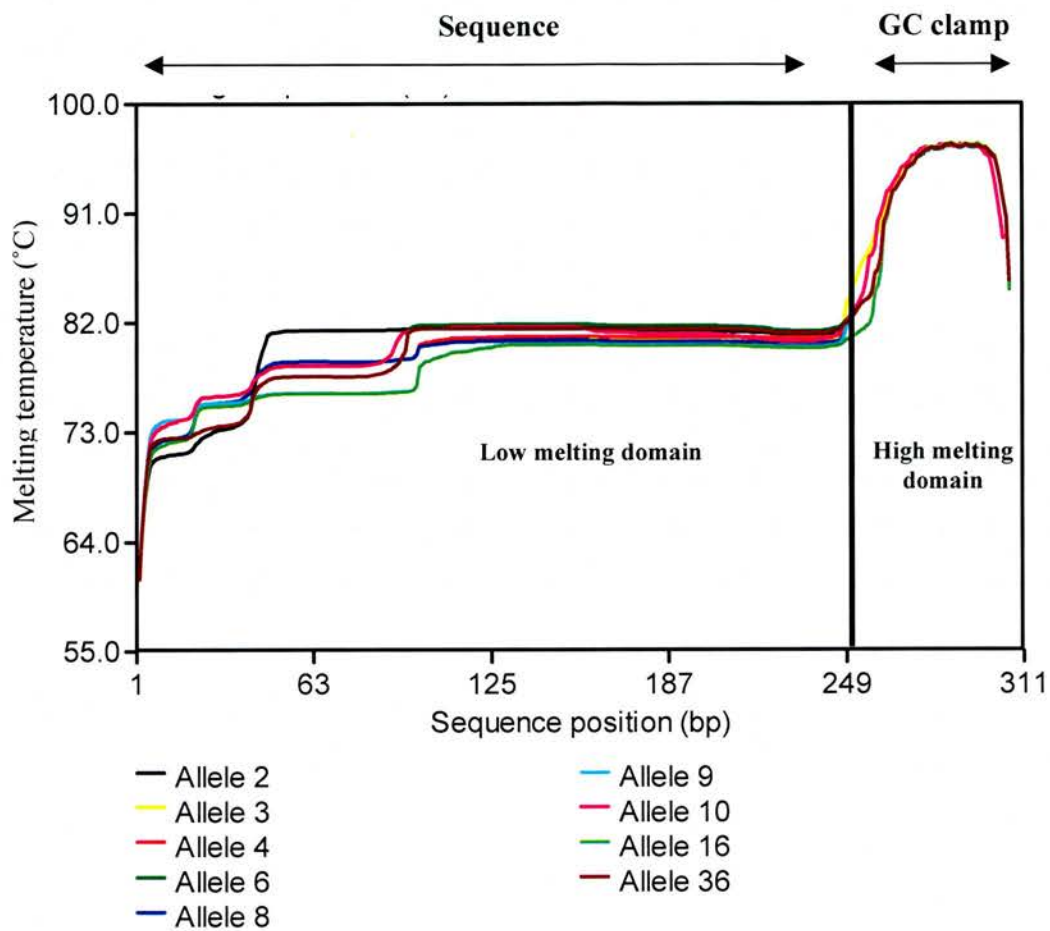

Figure 3.2 WinMelt™ graph of nine *Sus scrofa* DQB2 sequences with the addition of a 40 bp GC clamp at the 5′ end of the reverse primer.

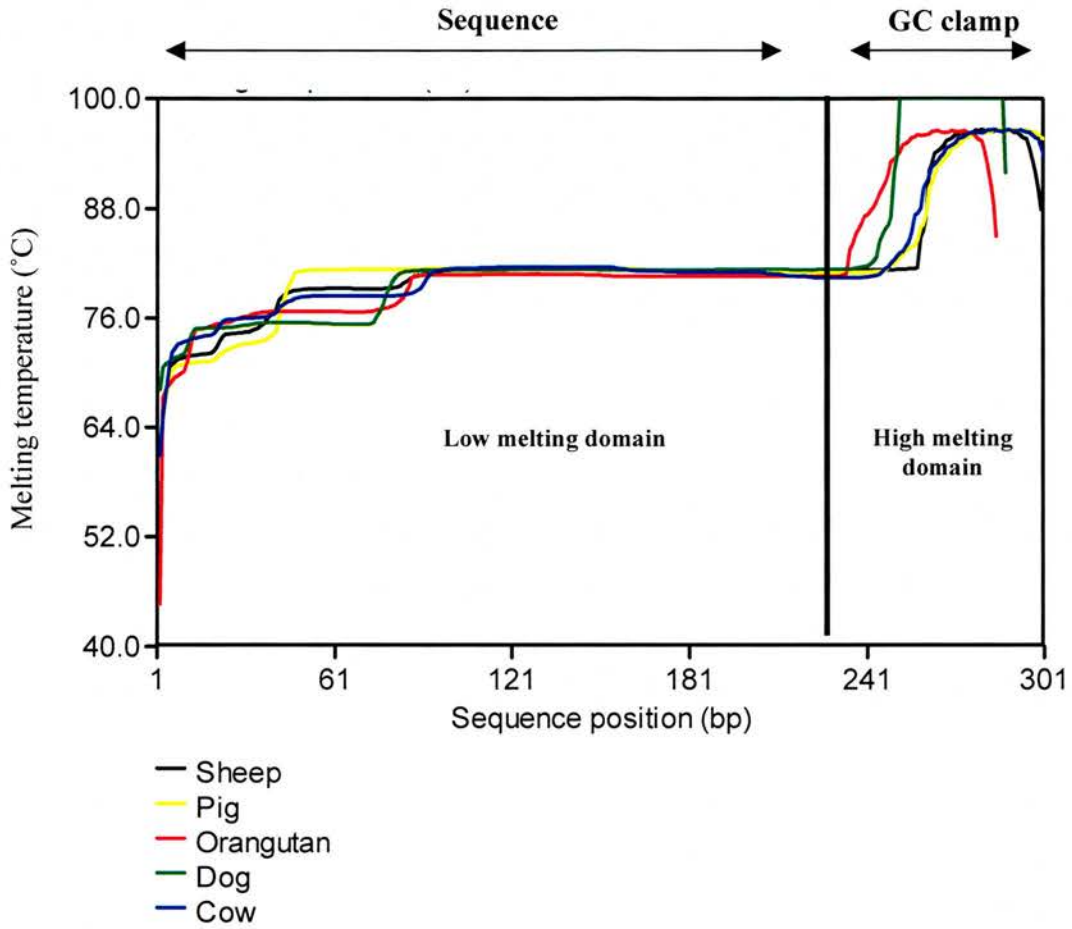

Figure 3.3 WinMelt™ graph of a single DQB2 sequence with the addition of a 40 bp GC clamp at the 5′ end of the reverse primer from a sheep, cow, pig, dog and orangutan.

3.2.4 DNA and primer quality

DNA quality was found to be particularly important for successful amplification of DQB2 alleles. DNA from eight grey seal samples that had been extracted using both Chelex and phenol-chloroform protocols were amplified using PCR. Initially, DNA that had been extracted using both methods yielded similar amounts of PCR product. However, storage of Chelex extracted samples for longer than a few months as well as repeated use of these samples resulted in a rapid decline in the number of samples in which PCR was effective, and decreased the yield obtained. After one year none of the eight test Chelex extracted samples could be amplified using PCR. In contrast, all eight phenol-chloroform extracted samples could be amplified using PCR procedures outlined in section 3.2.2. Hence, all samples were subsequently phenol-chloroform extracted. In addition, samples that were old (usually more than four years) and in particular those from dead pups yielded a lower success rate for PCR amplification. Degradation of these samples over time and/or poor sample preservation may have been the problem.

As with DNA quality, the quality of the primer affected the success of the PCR reaction and the effectiveness of the GC clamp when running samples through a parallel DGGE. Primers purified using standard desalting had a low PCR success rate. When PCR was successful, the amount of product yielded was often approximately half as much as could be obtained from DNA amplified using primers purified with high performance liquid chromatography (HPLC). In addition, desalted primers decreased the effectiveness of the GC clamp, resulting in bands appearing fuzzy once the product had been run using parallel DGGE. Hence, PCR amplification was conducted using HPLC purified samples, which increased PCR success and improved the resolution of bands on a parallel DGGE.

3.3 Perpendicular DGGE

Following PCR optimisation and the correct placement of the GC clamp, the next stage in optimisation of DGGE consists of determining the denaturing conditions under which alleles separate. The first stage involves running GC clamped PCR product on a perpendicular gradient gel, in which the gradient is perpendicular to the electric field. This method typically uses a very broad range of denaturants, 0 to 80 % such that the separation of alleles can be visualised and the range of denaturants under which alleles separate can be determined. However, before this could be determined the optimal run time and amount of PCR product needed for alleles to be separated and visualised had to be calculated.

This was achieved by running a series of perpendicular gels (12 % bis-acrylamide (37.5:1)) that varied in duration of electrophoresis and volume of PCR product loaded. These perpendicular gels were run at a constant temperature of 60 °C, at 300 volts and with a denaturing gradient of 0 to 80 %. All perpendicular and parallel gels were run using a BIORAD D-GENE apparatus (BioRad, Richmond, CA). PCR volumes of 50, 100, 200 and 400 µl plus 60 µl of 2x loading dye (2 % bromophenol blue, 2 % xylene cyanol, 70 % glycerol and 26 % water) from one individual were run for 1, 1.5, 2, 2.5, 3 and 4 hours. Gels were stained using SYBR Gold as outlined in section 3.4 and visualised using ultraviolet light at a wavelength of 320 nm. Electrophoresis times of less than two hours did not allow the PCR product to migrate far enough into the gel, preventing clear visualisation of alleles. Electrophoresis time longer than three hours resulted in the PCR product migrating off the gel. In addition, perpendicular DGGE using PCR volumes less than 400 µl did not allow for clear visualisation of alleles. Hence, 400 µl of GC clamped PCR products from single grey seal and wild boar individuals were electrophoresed through a 12 % bis-acrylamide

(37.5:1) gel with a gradient of 0 to 80 % urea and formamide. The voltage was kept constant at 300 V and the duration of electrophoresis was 2.25 hours in 7 litres of 1 x TAE (2 M Tris-base, 1 M glacial acetic acid, 50 mM 0.5 M EDTA, pH 8.0) at 60 °C. Under these conditions, optimal allele separation for grey seal samples occurred at a denaturing gradient of 25 to 55 % (Figure 3.4) and for the European wild boar of 10 to 60 % (Figure 3.5).

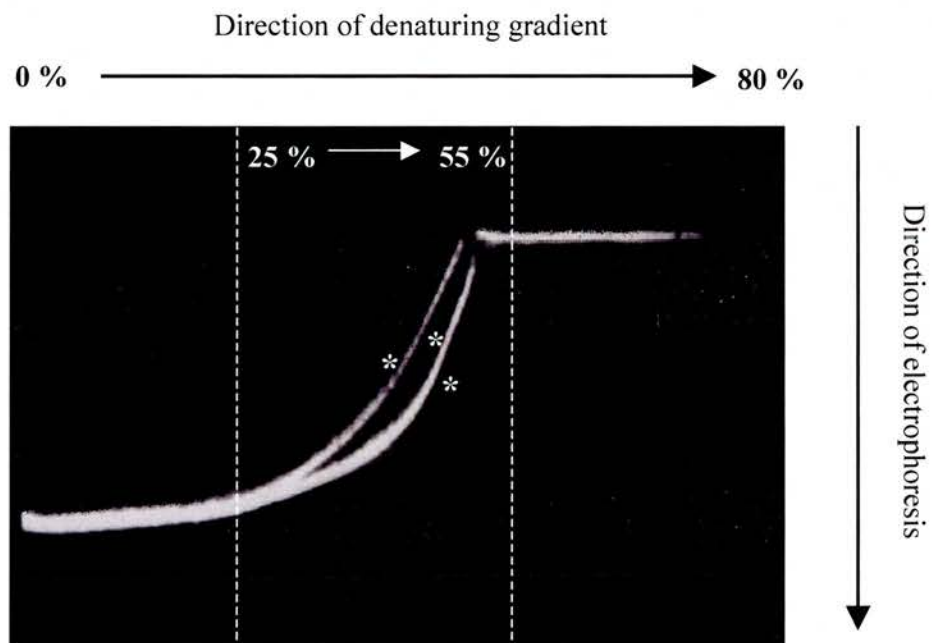

Figure 3.4 Perpendicular DGGE of GC clamped DQB2 PCR product from a single grey seal. The denaturing gradient is from 0 to 80 % and separation of alleles can be seen at a gradient of 25 to 55 %. Alleles detected on this gel are indicated with *.

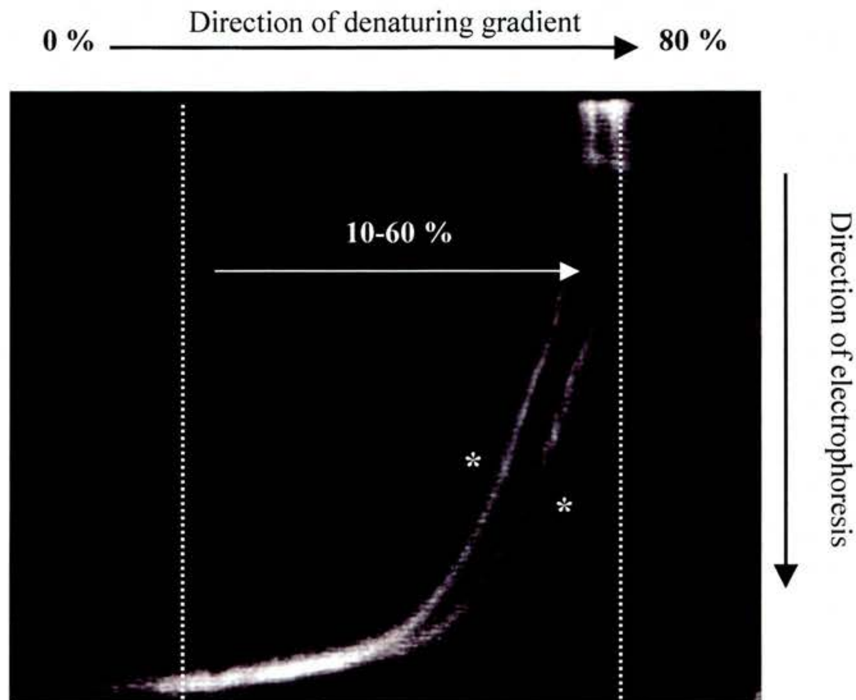

Figure 3.5 Perpendicular DGGE of GC clamped DQB2 PCR product from a single European wild boar. The denaturing gradient is from 0 to 80 % and separation of alleles can be seen at a gradient of approximately 10 to 60 %. Alleles detected on this gel are indicated with *.

3.4 Gel stains

Recommended gel stains for the visualisation of DQB2 PCR product on perpendicular and parallel DGGE included either a buffer bath of 1 x TAE containing 0.5 μg/ml ethidium bromide (BIORAD catalogue) or silver staining (Knapp et al. 1997b). To determine which stain produced the clearest visualisation of bands, two parallel DGGEs were conducted using the same eight samples. One parallel DGGE was stained using ethidium bromide, the other was silver stained. Comparison of the two gels revealed that ethidium bromide was not sensitive enough and silver staining, when it did work, was extremely time consuming, taking as long as two hours to complete. Instead I tested a new stain, SYBR® Gold nucleic acid gel stain (Molecular Probes) diluted to a

concentration of 1 x in a buffer bath of TAE (40 mM Tris-acetate, 1 mM EDTA, pH 7.5-8.0) at a pH of 7.0 to 8.5. Product was visualised under UV light at a wavelength of 320 nm. This method was quick, gels were placed in the staining bath for 10 to 30 minutes, the staining solution could be re-used up to three times, and was ten times more sensitive than ethidium bromide.

3.5 Parallel DGGE

3.5.1 Time series DGGE

The final step in optimisation of DGGE is to produce a parallel denaturing gradient gel using the denaturing conditions for allele separation determined from the perpendicular DGGE. Parallel DGGE involves producing the denaturing gradient parallel to the electric field. In doing so, 16 individuals can be run on a single gel (as opposed to one individual using perpendicular DGGE), and DQB2 alleles can be visualised as a series of bands. However, in order to determine optimal electrophoresis time for maximal allele separation, a time series parallel DGGE was conducted. This involved loading 35 to 40 μl of GC clamped PCR product and 15 μl of 2 x loading dye from the same four samples at intervals of half an hour starting at 3.5 hours and ending at 5 hours. Run time was considered optimal as soon as clear visualisation of all alleles was possible. Grey seal DQB2 PCR product from four individuals was run at 60 °C at 300 V on a 12 % bis-acrylamide gel (37.5:1) with a denaturing gradient of 25 to 55 % in 1 x TAE buffer. Gels were stained using SYBR Gold and visualised using UV light at a wavelength of 320 nm. Under these conditions all bands could be clearly visualised after five hours of electrophoresis (Figure 3.6). Wild boar DQB2 PCR product was run under the same conditions but with a denaturing gradient of 10 to 60 %. Optimal allele separation in the wild boar occurred after 4.5 to 5 hours of electrophoresis (Figure 3.7).

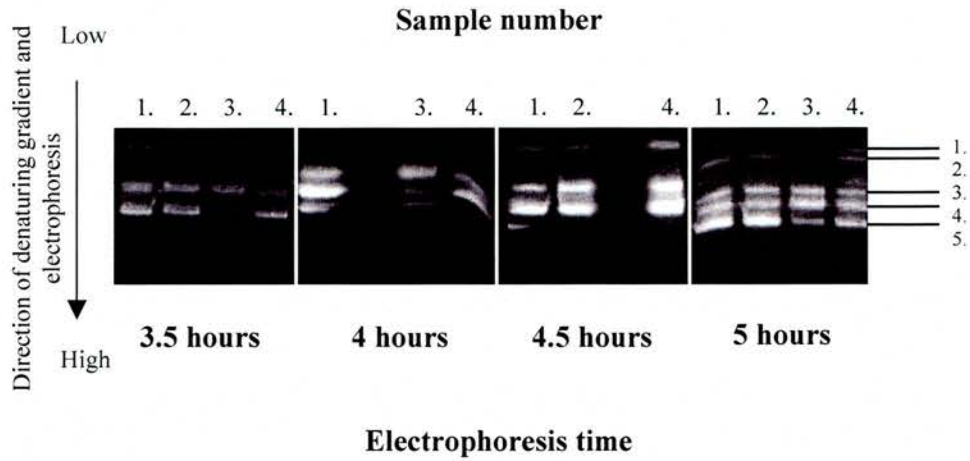

Figure 3.6 Time series parallel denaturing gradient gel of DQB2 PCR product from four grey seals. The denaturing gradient is from 25 to 55 % Alleles 1 to 5 are indicated for sample four after five hours of electrophoresis.

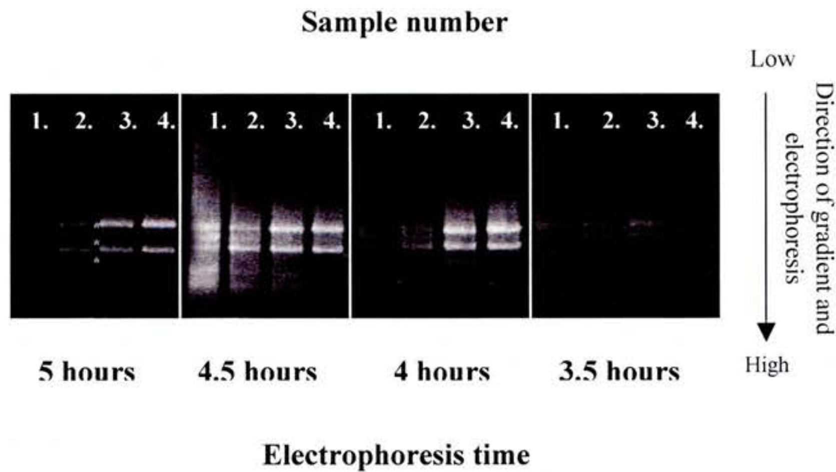

Figure 3.7 Time series parallel denaturing gradient gel of DQB2 PCR product from four European wild boars. The denaturing gradient is from 10 to 60 %. Each allele in sample two is indicated with a * after 5 hours of electrophoresis.

To determine if a longer period of electrophoresis increased band separation for both species, a second parallel gel was run using the same four samples loaded at intervals of half an hour starting at four and a half hours and ending at six hours. Electrophoresis time greater than five hours did not improve allele separation. However, in the grey seal, despite increased electrophoresis time, which can aid in allele separation, alleles appeared too close together, reducing the ability for individual alleles to be identified. Therefore, the denaturing gradient was decreased from 25 to 55 % to 40 to 55 %. This resulted in clearer visualisation of bands using the same run conditions as outlined above.

In the wild boar, parallel DGGE using a denaturing gradient of 10 to 60% also produced alleles that were too close together and bands could not be clearly resolved (Figure 3.8). In order to determine if each band represented a single allele, direct sequencing was carried out (see section 3.6). This revealed that in some cases a single band contained more than one allele. To separate alleles more effectively, the gradient was decreased to 30 to 55 %, which produced bands containing a single allele (Figure 3.9). This was confirmed by direct sequencing.

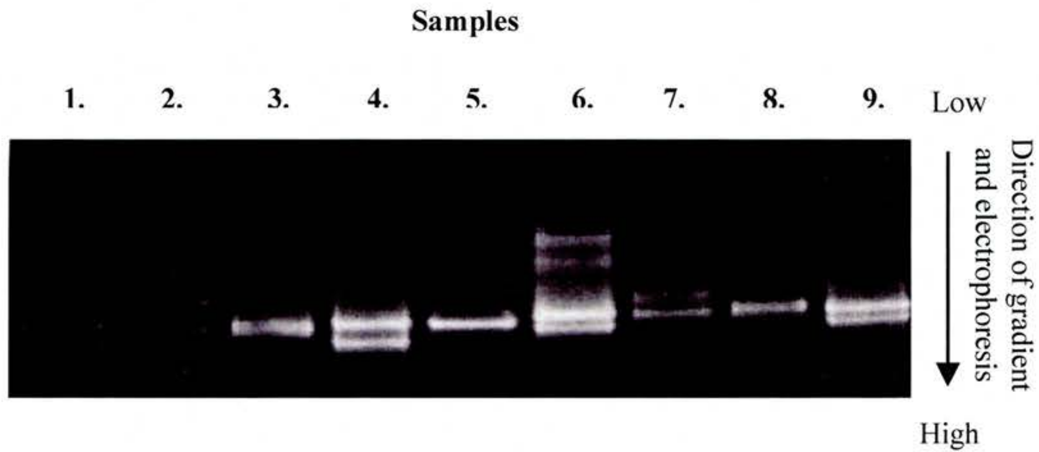

Figure 3.8 Parallel denaturing gradient gel of DQB2 PCR product from nine European wild boars. The denaturing gradient is from 10 to 60 % in the direction of electrophoresis.

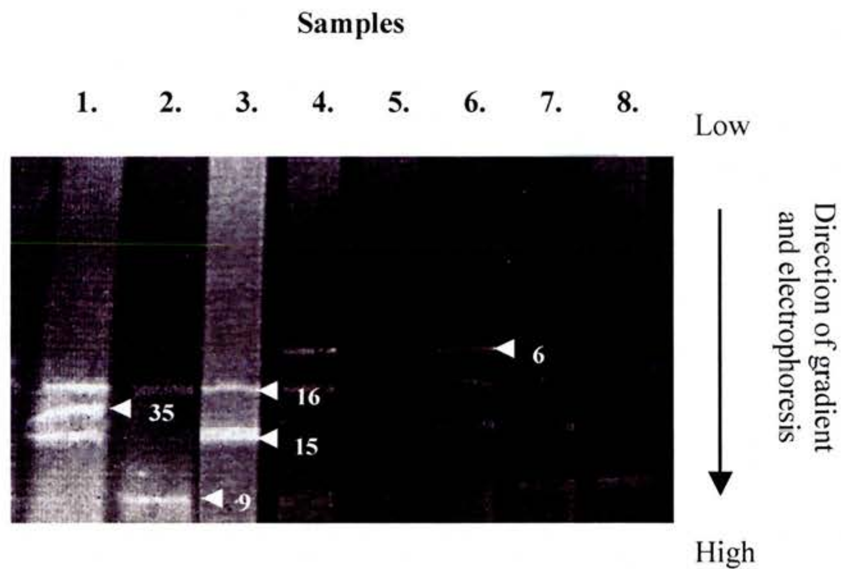

Figure 3.9 Parallel denaturing gradient gel of DQB2 PCR product from nine European wild boars. The denaturing gradient is from 30 to 55 % in the direction of electrophoresis. Alleles 6, 16, 35, 15 and 9 are labelled.

3.6 Direct sequencing

To confirm that each band on a parallel DGGE represented a single allele, direct sequencing was conducted. The section of gel containing a band was removed using a scalpel and then placed in an eppendorf containing 50 μl of deionized water, and incubated at 37 °C for 30 minutes. 2 μl of elute was then used in a PCR reaction under conditions outlined in section 3.2.2. To confirm successful amplification and remove primer dimer the PCR product was run on a 1.5 % agarose gel at 100 V for 30 minutes. PCR product was visualised under ultraviolet light at a wavelength of 320 nm and bands excised using a scalpel. Gel plugs were then placed in an eppendorf and PCR product extracted using a QIAquick® Agarose Gel extraction kit according to the manufacturer's instructions. The resulting product was concentrated to a volume of 4 μl by adding 10 % 3 M NaAC and 2.5 x 95 % ethanol. All of the concentrated PCR product was then used in a 15 μl sequencing reaction containing 0.8 μM M13 forward primer and 2 μl sequencing mix (provided by the Department of Genetics, University of Cambridge) following the sequencing cycling parameters detailed in Table 3.5. All reactions were overlaid with 10 μl mineral oil. The above procedure was repeated using the reverse M13 primer to provide sequences in both directions, allowing sequences to be checked by eye for correct assignment of base pairs using Chromas version 1.45 software.

Following PCR, the resulting product was separated from the mineral oil on parafilm and transferred to a 1 ml eppendorf to which 5 μl of deionised water and 80 μl of 75 % isopropanol (2-propane) was added. Tubes were left at room temperature for 15 minutes to precipitate out the extension products. PCR products were collected by centrifuging the tubes at 13,000 rpm for 20 minutes. Remaining liquid was aspirated off, leaving the pellet dry. The pellet was cleaned by rinsing it with 250 μl of 75 %

isopropanol (2-propane) and centrifuging at 13,000 rpm for five minutes. The supernatant was then aspirated off, the pellet dried in a vacuum centrifuge and then resuspended in 6 μl of 5:1 deionised formamide: 25 mM EDTA and electrophoresed on a 4 % polyacrylamide gel using an ABI 377 automated sequencer.

Table 3.5 Cycle sequencing parameters for sequencing of DQB2 alleles.

Step	Process	Temperature (°C)	Time (Seconds)	Number of Cycles
1	Denaturation	96	150	1
2	Denaturation	50	10	24
	Primer annealing	62	240	
	Extension	96	30	
3	Extension	50	10	1
	Extension	62	240	

3.7 Cloning

In addition to obtaining sequences from each gel plug, DQB2 PCR product from four grey seals and six wild boars were cloned to ensure that all alleles were being identified using DGGE and to produce clones of each allele that could be run as a ladder with each parallel DGGE. Direct sequencing from each gel plug in order to produce a standard ladder is problematic. This is because the amount of elute available is limited, approximately 50 μl, and does not provide enough 'stock' solution from which multiple PCRs can be conducted over the course of a project. This differs from colonies that can be cultured to provide a large amount of plasmid DNA containing the insert of each allele. Hence, colonies which contained DQB2 inserts that represented each of the alleles identified for both species were amplified using PCR and pooled together to produce a standard ladder to be run with each parallel DGGE.

3.7.1 PCR clean up and ligation

100 µl of DQB2 PCR product from four grey seals and six wild boars were purified to remove excess primer dimer and other impurities using QIAquick™ PCR Purification Kit (Qiagen). Purified PCR fragments were ligated into a Promega pGem®-T Easy Vector. Ligations involved 5 µl of 2 x Rapid Ligation Buffer (60 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10 % polyethylene glycol), 1 µl T4 DNA ligase, 1 µl of pGEM®-T Easy Vector (total concentration 50 ng), 4 µl of purified PCR product and 1 µl of T4 ligase (3 Weiss units). The ligation reaction was gently mixed and incubated at 4 °C overnight.

3.7.2 Transformation of competent cells and colony screening

Transformation reactions contained 5 µl of ligation mix plus 50 µl of DH5α™ Library Efficiency Competent Cells (Promega) which had been defrosted on ice. Reactions were incubated for 30 minutes on ice then heat shocked in a 37 °C water bath for 20 seconds. The reaction mix was then placed on ice for another two minutes before adding 900 µl of S.O.C. medium (1.9 % Bacto-typtone, 0.19 % Bacto-yeast, 95 mM NaCl, 20 mM glucose) at 37 °C. Tubes were placed in a shaking incubator at 225 rpm for one hour at 37 °C to allow expression of antibiotic resistance. 100 µl of reaction mix was spread onto LB agar (1.9 % Bacto-typtone, 0.19 % Bacto-yeast, 95 mM NaCl, 2 % agar) plates containing 10 mg/ml carbicillin and 0.6 % X-gal. Plates were inverted and incubated at 37 °C for 12 hours.

In order to determine which colonies contained PCR inserts, blue/white screening was conducted. Successful cloning of an insert into a vector interrupts the coding sequence and recombinant clones can be identified by colour. Blue colonies do

not contain an insert and white colonies contain the PCR insert of interest. Hence, white colonies were selected using sterile wooden toothpicks and transferred to a sterile tube containing 2 ml of LB with carbicillin (100 mg/ml). Tubes were placed in a shaking incubator for 12 hours at 225 rpm at 37 °C to increase the number of colonies transformed.

3.7.3 Preparation of plasmid DNA by alkaline lysis with SDS: Minipreparation

Following incubation of white colonies in a solution of LB, 1.5 ml of culture was transferred to an eppendorf tube and centrifuged at 13,000 for 30 seconds. Supernatant was removed and the remaining pellet left as dry as possible. The pellet was resuspended in 100 µl of alkaline lysis solution I (50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0)) and let to sit for five minutes. 200 µl of freshly prepared alkaline lysis solution II (0.2 M NaOH, 1 % SDS) and 150 µl of solution III (3 M potassium acetate, 5 M glacial acetic acid) was added to the solution, rapidly inverted five times and then put on ice for five minutes. The bacterial lysate was then centrifuged at 13,000 rpm for ten minutes and the supernatant transferred to a fresh tube. Plasmid DNA was then precipitated out from the remaining supernatant by adding two volumes of 95 % ethanol and 10 % of the supernatant volume of 3 M NaAC (pH 4.6) and freezing at –70 °C for 30 minutes. Precipitated plasmid DNA was collected by centrifuging at 13,000 rpm for ten minutes. The supernatant was then removed and 0.5 ml of 70 % ethanol added before centrifuging for another five minutes at maximum speed. The supernatant was then discarded and the pellet dried in a 37 °C oven for 30 minutes. The pellet, containing the plasmid DNA, was dissolved in 40 µl of water containing 20 µg/ml of RNAse A.

3.7.4 Fragment recovery and sequencing

In order to determine if the plasmid DNA contained a fragment of the DQB2 region, 6 μl of plasmid DNA was digested in 11 μl of ultra pure water, 2 μl of 10 x Buffer H and 1 μl of EcoR 1. Reactions were placed in a 37 °C oven for four hours. 10 μl of digested product was then loaded onto a 1.5 % agarose gel with a 100 bp ladder. If the fragment was of the same length as the DQB2 insert, the plasmid was used for sequencing.

Of those plasmids containing fragments of the appropriate size sequencing reactions were performed on plasmid DNA. Sequencing reactions were conducted in a final volume of 15 μl and contained 250 ng plasmid DNA, 0.8 μM M13 forward primer and 2 μl sequencing mix (supplied by the Department of Genetics, Cambridge University). All reactions were overlaid with 10 μl mineral oil, followed by sequencing using the cycling parameters detailed in Table 3.5. This was repeated using the reverse M13 primer to provide two sequences per clone which could be then checked visually using Chromas software for correct assignment of base pairs and to ensure that both the forward and reverse sequence were in agreement. Bases that had been misidentified by the automatic sequencing program were corrected manually.

A total of 28 grey seal clones were sequenced in this way to provide three complete full length (271 bp) sequences for each of the five alleles identified in this study. Thirty-four wild boar clones were sequenced to provide three full length (256 bp) sequences for each of the five alleles identified. Some clones exhibited three to eight occasional differences in the position of particular base pairs. This is to be expected because of occasional missincorporation of nucleotides by the *Taq* polymerase (Kobayashi et al. 1999). These differences could be manually checked by comparison of chromatograms of each sequence that had been sequenced from the forward and reverse primer using Chromas software. All wild boar and grey seal sequences

identified in this thesis were aligned using ClustalW software (see Table 3.10 and 3.11 for final alignment) (European Bioinformatics Institute; http:\\www.ebi.com).

Since published sequences for the European wild boar exist, the sequences obtained during this study were compared with those already published using the Basic Local Alignment Search Tool (BLAST) available at the National Centre for Biotechnology Information webpage (http://www.ncbi.nlm.nih.gov/BLAST) in order to determine which alleles had been amplified in this study. Using BLAST, the five alleles identified in this study included two from the MHC class II antigen (SLA-DQB) gene; alleles SLA-DQB*P16 (16) and SLA-DQB*P35 (35), and three from the MHC class II SLA-DQB beta-1 domain (SLA-DQB) gene; alleles SLA-DQB-S15 (15), SLA-DQB-S06 (06) and SLA-DQB-S09 (09).

Table 3.10 Sequence alignment of five grey seal alleles from the DQB2 region using ClustalW. A dash indicates the same nucleotide. Primers are indicated in bold.

Allele	Sequence 5′ - 3′
2	**TCGTGTACCA GTTTAAGGGC** GAGTGTCATT ATTCCAACGG GACGGAGCGG GTGCGGTTCC
4	---------- ---------- ---------- ---------- ---------- ----------
3	---------- ---------- -----CT-C- TCA------- ---------- ------C---
1	---------- ---------- -----CT--- TCA------- ---------- ------C---
5	---------- ---------- -----CT--- TCA------- ---------- ---T--C---
	10 20 30 40 50 60
2	TGGATAGGCA TTTCTATAAC GGGGAGGAGT TCGTGCGCTT CGACAGCGAC GTGGGGGAGT
4	---TC--AG- CA-----T-- ---------G A--------- ---------- ----------
3	---TC--ATG CA-------- ---------- A--------- ---------- ----------
1	--ACC--AT- CA-------- ---------- A--------- ---------- ----------
5	--ACC--AT- CA-------- ---------- A--------- ---------- ----------
	70 80 90 100 110 120
2	TCCGGCCGGT GACGGAGCTG GGGCGGCCGG ACGCTGAGTA CTGGAACCGC CAGAAGGACT
4	---------- ---------- ---------- ---------- ---------- ----------
3	---------- ---------- --------C- ---------- ---------- ---------A
1	A--------- ---------- ---------- ---------- -------A-- ---------A
5	A--------- ---------- ---------- ---------- -------A-- ---------A
	130 140 150 160 170 180
2	TCATGGAGCG GAAGCGGGCC GCGGTGGACA GGCTGTGCAG ACACAACTAC GGGGTGGGTG
4	--------GA --C------- ---------- C-G------- ---------- -------T--
3	--G------- --C------- -A-------- C-G------- ---------- -------T--
1	--G------- --C------- -A-------- C-G------- -A-------T ---T-T-AG-
5	--G------- --C------- -A-------- C-G------- -A-------T ---T-T-AG-
	190 200 210 220 230 240
2	AGAGCTTCAC GGTG**CAGCGG CGAGGTGAGC G**
4	---------- ---------- ---------- -
3	---------- ---------- ---------- -
1	----AACG-- CT-------- ---------- -
5	----AACG-- CT-------- ---------- -
	250 260 270

Table 3.11 Sequence alignment of five European wild boar alleles from the DQB2 region using ClustalW. A dash indicates the same nucleotide. Primers are excluded.

Allele	Sequence 5′ - 3′					
35	GATTTCGTGT	ACCAGTTTAA	GGGCGAGTGC	TACTTCTACA	ACGGAACGCA	GCGGGTGCGG
15	----------	----------	----------	----------	----------	----------
16	----------	----------	----------	----------	----------	-------T--
06	----------	----------	-TT-------	-------T--	----------	----------
09	----------	----------	-TT-------	----------	----------	----------
	10	20	30	40	50	60
35	CTCGTGGCCA	GATACATCTA	CAACCAGGAG	GAGCATTTGC	GCTTCGACAG	CGACGTGGGG
15	----------	----------	----------	----------	----------	----------
16	AG-----A--	----------	----------	---TT-----	----------	----A-----
06	---T--A---	----------	----------	-----CG---	----------	-A--------
09	----------	-G-GGG----	-----G----	-----CG---	----------	----------
	70	80	90	100	110	120
35	GAGTTCCGGG	CGGTGACCCC	GCTGGGGCGG	CCGGAGGCCG	ACTCCTGGAA	CAGCCAGAAG
15	----------	----------	----------	----------	----------	----------
16	----A-----	----------	----------	-----C----	---A--T---	-G--------
06	----A-----	----------	----------	-----C----	---A------	-G--------
09	----------	----------	----------	-----C----	---A------	-G--------
	130	140	150	160	170	180
35	GACGTCCTGG	AGCAGAAGCG	GGCCGAGCTG	GACACTGTGT	GCAAACACAA	CTACCAGATA
15	----------	----------	----------	----------	----------	----------
16	--G-C-----	----------	----------	----------	----------	----------
06	----------	------C---	----------	----------	----------	----------
09	--G-------	----------	----------	-----G----	----------	----------
	190	200	210	220	230	240
35	GAGGAAGGCA	CGACTC				
15	----------	----C-				
16	----------	------				
06	----------	----C-				
09	----------	----C-				
	250	256				

3.8 Ladder construction

3.8.1 Colony boils and PCR

Minipreparation and digestion of a single plasmid colony followed by electrophoresis of resulting plasmid DNA and the cloned fragment on an agarose gel are useful techniques to determine if a plasmid contains an insert of the appropriate length. However, this method can be slow when screening a large number of colonies. In order to determine if a colony contained the correct insert quickly and without the need for minipreparation and digestion, a colony boil followed by PCR with the original primers was conducted for each white colony. Each white colony was picked with a sterile wooden toothpick and shaken in a 1 ml tube containing 200 μl of 1 x TE for ten seconds. Each tube was then incubated ("boiled") at 100 °C for ten minutes. Following incubation, PCR was conducted on approximately 40 ng of plasmid DNA, 1.5 mM $MgCl_2$, 10 mM Tris, 50 mM KCl, 100 μM dTTP, dATP, dCTP, 1U *Taq* polymerase and 30 pmol of each primer using the cycling parameters outlined in Table 3.12. To determine which colonies contained the correct insert, 8 μl of PCR product and 3 μl of 6 x loading dye was run on a 1.5 % agarose gel with a 100 bp standard ladder (provided by Promega) at 100 V for 30 minutes.

Table 3.12 PCR amplification cycling parameters for colony boil samples.

Step	Process	Temperature (°C)	Time (Seconds)	Number of Cycles
1	Denaturation	96	350	1
2	Denaturation	94	45	30
	Primer annealing	51	60	
	Extension	72	120	
3	Extension	72	600	1

3.8.2 Colony identification

To determine which alleles were present in each of the colony boils, 5 to 10 μl of PCR product was run on a parallel gradient gel following the conditions outlined in section 3.5. This allowed a single colony representing each allele to be selected and colony boils and PCR to be conducted as outlined above. The resulting PCR product was then pooled to construct a ladder to be run with each parallel DGGE (for example see Figure 3.10).

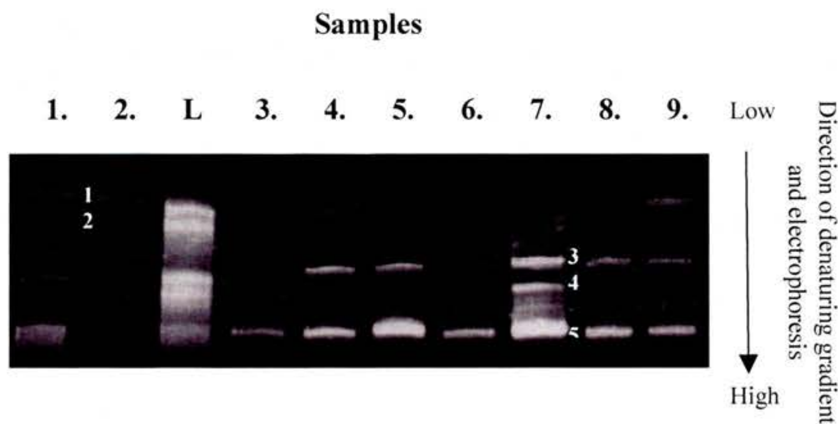

Figure 3.10 A parallel denaturing gradient gel (40 to 55 %) of DQB2 PCR product from nine grey seals. The standard ladder (L) that is run with each gel is indicated, as are the five alleles.

3.9 Phylogenetic analysis

Since the primers I used for both species were expected to amplify exon 2 sequences from multiple DQB loci, subsequent attribution of sequences to particular loci was performed using maximum-parsimony analysis (Swafford & Stewart 1987), and neighbour-joining analysis (Saitou & Nei 1987) using MEGA 2.1 (Molecular Evolutionary Genetic Analysis) (Kumar et al. 2001). For maximum parsimony analysis a consensus tree was derived by bootstrap analysis with 1,000 replications. For

neighbour-joining analysis, genetic distances were derived using the Jukes-Cantor correction and a bootstrap analysis with 1,000 replications was used. A single allele from the MHC class II DQB region of exon 2 in *Canis familiaris* (DQB1*00201 allele exon 2 and partial cds, Accession number AF016908) obtained from GENBANK was used as an outgroup in all cases.

Using the wild boar and grey seal sequences identified in this study I identified four and three potential linkage groups or loci for each species respectively (Figure 3.11). In the grey seal, allele 3 appears to be the result of a duplication event and is very closely linked to locus group L1 (Figure 3.12). Grey seal alleles 2 and 4 link to form locus L1 and alleles 1 and 5 link to form L3 (Figure 3.12). For the wild boar alleles 15 and 35 link to form group L1, alleles 16, 9 and 6 are in their own groups, L2, L3 and L4 respectively.

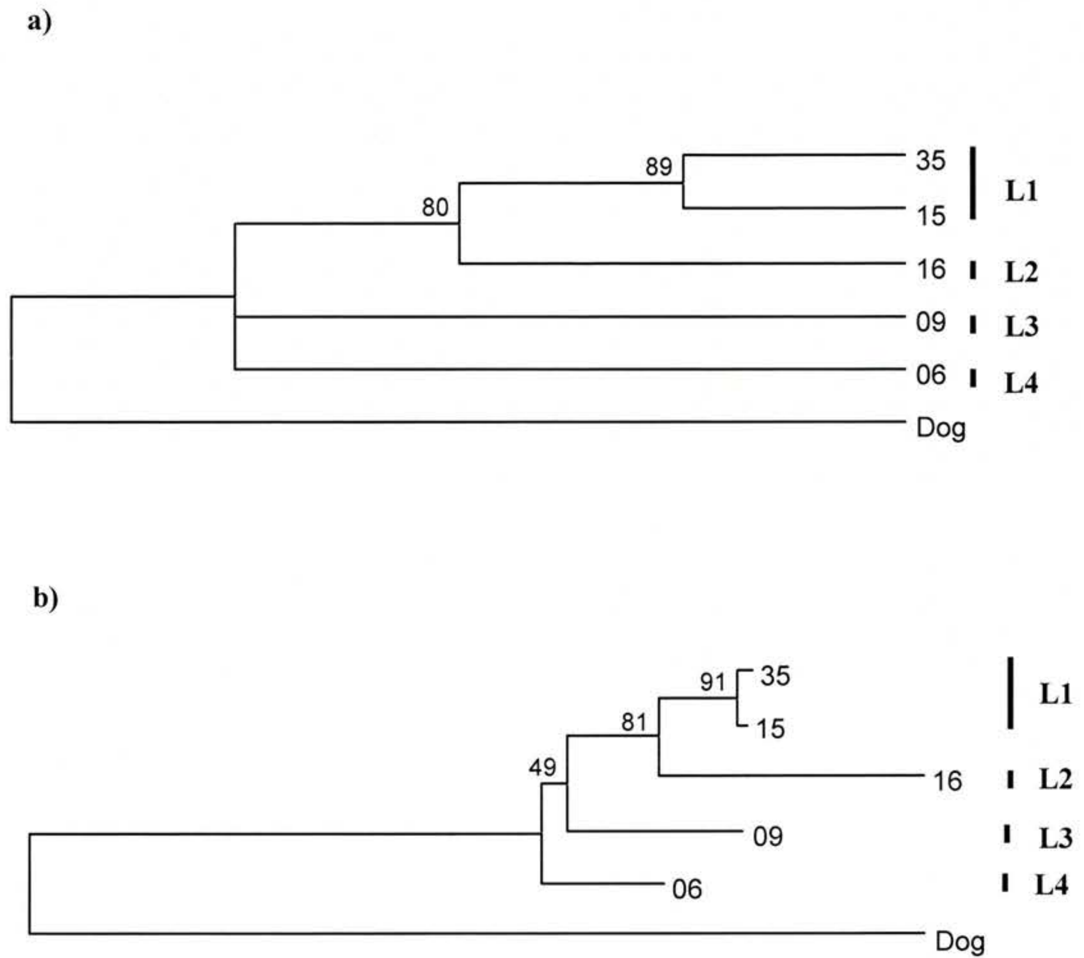

Figure 3.11 **a)** Maximum-parsimony consensus tree based on 1,000 bootstrap replications and **b)** neighbour-joining tree based on 1,000 bootstrap replications of DQB2 alleles in wild boar. A single class II MHC DQB allele from exon 2 in the dog (*Canis familiaris*) is used as an outgroup. Bootstrap values are indicated at the beginning of each branch. Loci are indicated as L1, L2 and L3.

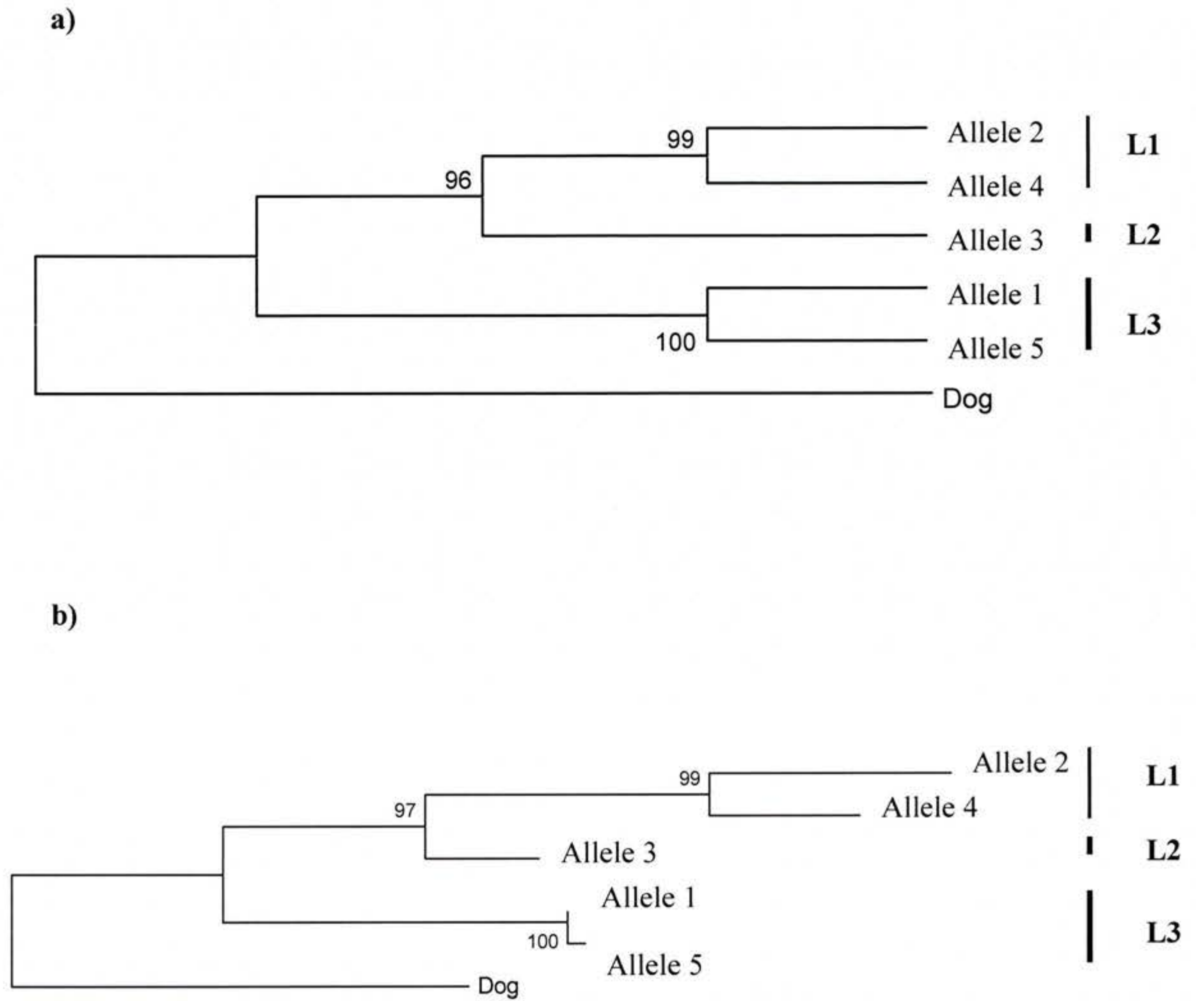

Figure 3.12 **a)** Maximum-parsimony consensus tree based on 1,000 bootstrap replications and **b)** neighbour-joining tree based on 1,000 bootstrap replications of DQB2 alleles in the grey seal. A single class II MHC DQB allele from exon 2 in the dog (*Canis familiaris*) is used as an outgroup. Bootstrap values are indicated at the beginning of each branch. Loci are indicated as L1, L2 and L3.

3.10 Conclusions

This chapter describes the identification and characterisation of DQB2 alleles in the grey seal and European wild boar. For the grey seal five alleles were identified. This finding is similar to levels of DQB2 diversity documented in other seal species where diversity ranges from two to eight alleles (Slade 1992; Hoelzel et al. 1999; Lento et al. 2003). These alleles form three linkage groups which were treated as separate loci in the subsequent analyses. Similarly, for the wild boar five DQB2 alleles were identified and these alleles form four linkage groups which were also treated as separate loci.

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CHAPTER 4

The relative importance of the DQB2 region on mating strategy in the grey seal

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4.1 Introduction

Female mammals generally invest more resources in their offspring than males (Trivers 1972). Consequently, males are most likely to increase their fitness by monopolising females either by aggressively excluding other males (Pemberton et al. 1992) and/or by developing elaborate ornamentation to attract females (Hamilton & Zuk 1982). Such strategies are exemplified by the antlers grown by male deer and the amazing plumage of male birds of paradise. In contrast, females by and large cannot increase the number of offspring they raise and therefore should be selective and choose a male that provides the best resources, genes, or both (Trivers 1972). Females will aim to increase the fitness of their offspring either by choosing 'good genes', or by selecting a mate which will increase the overall genetic heterozygosity of the offspring (Trivers 1972). Hence, in order to produce offspring of a higher fitness, females need to be able to assess not only the quality of a mate's genes, but also the extent to which they complement their own in order to produce offspring that are maximally heterozygous.

One of the best examples of 'good genes' and heterozygosity driving mating preference involves the genes from the MHC. In particular, studies on mice have shown that females use MHC-dependent odour cues to detect the degree of MHC similarity of a potential mate (Yamazaki et al. 1976; Ehman & Scott 2001). For example, in house mice, *Mus musculus*, females preferentially mate with males whose MHC haplotype is different from their own (Egid & Brown 1989). Not only do females select MHC-dissimilar mates, they may also choose mates which carry a large number

of MHC alleles. To test this, Reusch et al. (2001) investigated the relative importance of an 'allele counting' strategy compared to a dissortative mating strategy in three-spined sticklebacks (*Gasterosteus aculeatus*). In this study, females preferred the odour of males which had a large number of MHC class-IIB alleles to that of males with fewer alleles, thereby supporting the 'allele counting' strategy (Reusch et al. 2001). As well as choosing MHC-dissimilar or diverse mates, avoidance of haplotypes that increase susceptibility to infection and disease may also occur. For example, in mice, not only can females detect how similar a male is in their MHC haplotype, females mate preferentially with, and prefer the odours of, non-parasitised males (Kavaliers & Colwell 1995a; 1995b; Ehman & Scott 2002). It could be suggested that male mice carrying particular haplotypes were more susceptible to infection and are therefore avoided by females as has been documented by Harf & Sommer (2005), who found that particular DRB alleles are important in determining parasite load in the hairy-footed gerbil (*Gerbillurus paeba*).

MHC-dependent mate choice could also function to avoid genome-wide inbreeding (Tregenza & Wedell 2000). Potts et al. (1994) investigated fitness in relation to inbreeding and MHC homozygosity in semi-natural populations of wild-derived house mice and revealed that there was a fitness decline, females produced fewer young and males were less likely to gain territories, associated with inbreeding, but none related to MHC homozygosity. They suggested that inbreeding avoidance may be the most important function of MHC-based mating preferences and may be the selective force diversifying MHC genes (Potts et al. 1994). However, this view is controversial and similar studies on other species are required to determine the relative importance of these two measures on mate choice.

Although numerous studies have investigated the influence of the MHC on mating preferences, the majority of these have focused on laboratory and semi-natural populations of house mice (Egid & Brown 1989; Eklund et al. 1991; Kavaliers & Colwell 1995a; Eklund 1997a; Ehman & Scott 2002). More recently, investigation of MHC-correlated mate choice has been extended to include laboratory studies on other taxa such as Atlantic salmon (*Salmo salar*) (Landry et al. 2001), three-spined sticklebacks (*Gasterosteus aculeatus*) (Milinski & Bakker 1990; Reusch et al. 2001) and human beings (Wedekind et al. 1995; Ober et al. 1997). However, very few studies have investigated MHC-dependent mating preferences in wild populations of vertebrates (for examples see Eklund 1997b and Paterson & Pemberton 1997). Work presented in this chapter aims to fill this gap using two wild populations of grey seal.

Studies investigating the mating behaviour in the grey seal have spanned 40 years (Boyd et al. 1962). Initially, such studies were based on behavioural data (Cameron 1969; Fogden 1971; Anderson et al. 1975; Pomeroy et al. 1994; Twiss et al. 1994) but more recently, molecular based studies have revealed that although polygyny is the dominant mating strategy, aquatic mating and some level of mate choice may also operate simultaneously within the colony (Amos et al. 1995; 2001b). Female mate choice in grey seals has been inferred from the presence of large numbers of full siblings within breeding colonies, where females are believed to be favouring previous parental combinations (Amos et al. 1995). Furthermore, a study by Amos et al. (2001b) found that maternal half-siblings who have different fathers are significantly more genetically diverse (measured using microsatellites) than would be expected from random mating. This led them to suggest that females are able to be selective and choose genetically diverse mates (Amos et al. 2001b).

The occurrence of both mate choice and polygyny in the grey seal provides an ideal system in which female choice can be investigated in relation to MHC variation. In addition, due to extensive studies on two populations on the Isle of May and North Rona over the last 20 years, a large and comprehensive dataset containing tissue and blood samples from mothers and their pups is available. Such data are usually difficult to obtain from most wild populations of large mammals.

In this chapter I investigate the influence of the MHC class II DQB exon 2 region (DQB2) on mating behaviour in the grey seal from two island populations, the Isle of May and North Rona. Firstly, I investigate if females choose DQB2-dissimilar males, and the extent to which this varies over consecutive pupping events on both islands. I also investigate the proportion of heterozygous and homozygous pups born in each pupping event. Here it is expected that because heterozygotes are believed to have a fitness advantage over homozygotes, females will chose males that are genetically dissimilar to themselves; that is, they will chose a male with whom they share the fewest alleles across loci (Mays & Hill 2004). By choosing genetically dissimilar males females may produce considerably more heterozygous offspring (Mays & Hill 2004).

Secondly, as well as choosing MHC-dissimilar mates, females may also choose males to increase DQB2 diversity in their offspring. In this chapter I investigate variation in DQB2 diversity in pups over consecutive pupping events. Thirdly, I test the hypothesis that females will choose males that have alleles or combinations of alleles that may increase fitness in their offspring by examining the proportion of each DQB2 allele in pups compared to mothers over consecutive pupping events.

4.2 Materials and Methods

4.2.1 Sampling methods

4.2.1.1 Focal females

I used an existing sample collection containing tissue and blood samples from mothers and their pups sampled over nine pupping seasons on the Isle of May and ten pupping seasons on North Rona. Both North Rona and the Isle of May populations contain a number of permanently marked adult female grey seals. These females were hot iron branded by previous researchers between 1986 and 1989 with individual letter/number ciphers on each flank. This method of individual identification has proved to be reliable, long lasting and easily visible in the field, even when weather conditions are poor. For each of these branded females, extensive data exists on choice of pupping site, date of arrival, pupping success, body condition, length, girth and weight. Tissue and blood samples collected in the past by other studies beginning in 1986 were used in this study. In 2002, I visited the Isle of May and worked with the Sea Mammal Research Unit (SMRU) to collect tissue samples and other body measurements from focal females and their pups. Collaborating with other researchers in this way reduces the frequency of human contact with the females and their pups for different purposes, reducing potential stress for the female and her pup, and disruption to the colony in general.

In order to obtain skin and/or blood samples from focal females, each female was anaesthetised two to three days after pupping with an intramuscular dose of either ketamine hydrochloride or zolazepam-tiletamine ('Zoletil', Vibac, UK) using darts fired from a blow pipe (Figure 4.1). Once sedated, the female was restrained using a net. Between 1986 and 1989, blood samples were collected from the extradural vein (lying

ventro-laterally to the spinal cord) and stored in an EDTA coated vacutainer (Isle of May, n = 28; North Rona, n = 27). In later years, skin samples were taken instead of blood, using a pair of pig ear-notching pliers (Isle of May, n = 20; North Rona, n = 0). All sites of skin and blood sampling were treated with oxytetracycline antibiotic spray. Females were monitored until they regained mobility and pups were placed in close contact with their mother. Skin samples were stored in 20 % DMSO saturated with salt and frozen at –70 °C (Amos & Hoelzel 1991). Samples collected in 2002 were preserved in 95% ethanol and stored at room temperature. All procedures involving the capture and handling of females and pups during this study were carried out under Home Office licence PIL 80/390.

Figure 4.1 Blow dart pipe aimed at a female grey seal for administration of an intramuscular dose of either ketamine hydrochloride or zolazepam-tiletamine.

4.2.1.2 Pups from focal females

Pups, unlike adult females, do not need to be sedated in order to collect tissue samples. Instead, each pup associated with a focal female was restrained in a large bag while the mother was sedated (Figure 4.2). A blood sample was taken from the vascular plexus (between the digits at the point of origin of the hind flipper) from pups sampled between 1986 and 1989 (Isle of May, n = 54; North Rona, n = 110). In subsequent years, a skin sample was taken from the interdigital webbing of the hind flipper using a pair of pig ear-notching pliers, and the samples stored as above (Isle of May n = 161; North Rona n = 130). All DNA extraction, PCR and genotyping techniques for tissue and blood samples collected from focal females and their pups were conducted following procedures outlined in chapter three.

Figure 4.2 A single pup from a focal female being weighed inside a pup weighing bag using balance scales.

□4.2.2 Data analysis

For each mother-pup pair the number of pup DQB2 alleles that differed from the female haplotype were counted to provide an estimate of DQB2-dissimilarity between females and their pups. To examine if pups differed from their mother in the DQB2 alleles they carried and if this varied over consecutive pupping events I fitted a GLM with pupping order (primiparous versus second, third etc born pups) as a categorical factor and the number of DQB2 allele differences as the response variable. Variation in the proportion of homozygous and heterozygous pups on each island was analysed using chi-squared analysis, the sixth and seventh born (North Rona) and seventh and eighth born (Isle of May) pup categories were pooled due to low sample size (Sokal & Rohlf 1995). Overall levels of heterozygosity and homozygosity in pups from each island were analysed using chi-squared tests with Yates correction. To determine if DQB2 diversity (the number of DQB2 alleles carried by an individual) in mothers and pups varied over consecutive pupping events, I fitted a GLM with pupping order as the categorical factor and DQB2 diversity as the response variable. The proportion of each DQB2 allele in pups compared to mothers was compared using chi-squared analysis, with the last two pupping event categories on each island pooled due to low sample size (Sokal & Rohlf 1995).

4.3 Results

4.3.1 Do females choose DQB2-dissimilar mates?

If female grey seals actively choose DQB2-dissimilar mates, ie. participate in some form of mate choice, one would expect pups to have an MHC haplotype that is maximally dissimilar to that of the female. Here, I compare the number of pup DQB2 alleles that differ from the female haplotype on the Isle of May and North Rona. On the Isle of May there was a significant difference in the number of DQB2 alleles in pups that differed from the maternal haplotype over consecutive pupping events ($F_{1,91} = 7.93$, $P < 0.001$). Primiparous pups on the Isle of May had few DQB2 alleles that differed from the female haplotype (mean number of alleles = 1.11, S.E. = 0.21) yet by the sixth pupping event the mean number of alleles that differed from the female haplotype was as high as 2.5 (S.E. = 0.5) (Figure 4.3). There was also a gradual increase in the number of pup DQB2 allele differences from the female haplotype from the first to sixth born pup (Figure 4.3). Similarly, on North Rona there was a significant difference in the number of pup DQB2 alleles that differed from the female haplotype ($F_{1,104} = 6.73$, $P < 0.001$). As with the Isle of May, primiparous pups did not differ significantly from the female haplotype (mean number of alleles = 1.17, S.E. = 0.24) and there was an upward trend of increasing dissimilarity (with the exception of pup event 4) until pup five (Figure 4.3). However, after pup 5 (mean number of alleles = 2.3, S.E. = 0.33) levels of DQB2 dissimilarity declined (Figure 4.3).

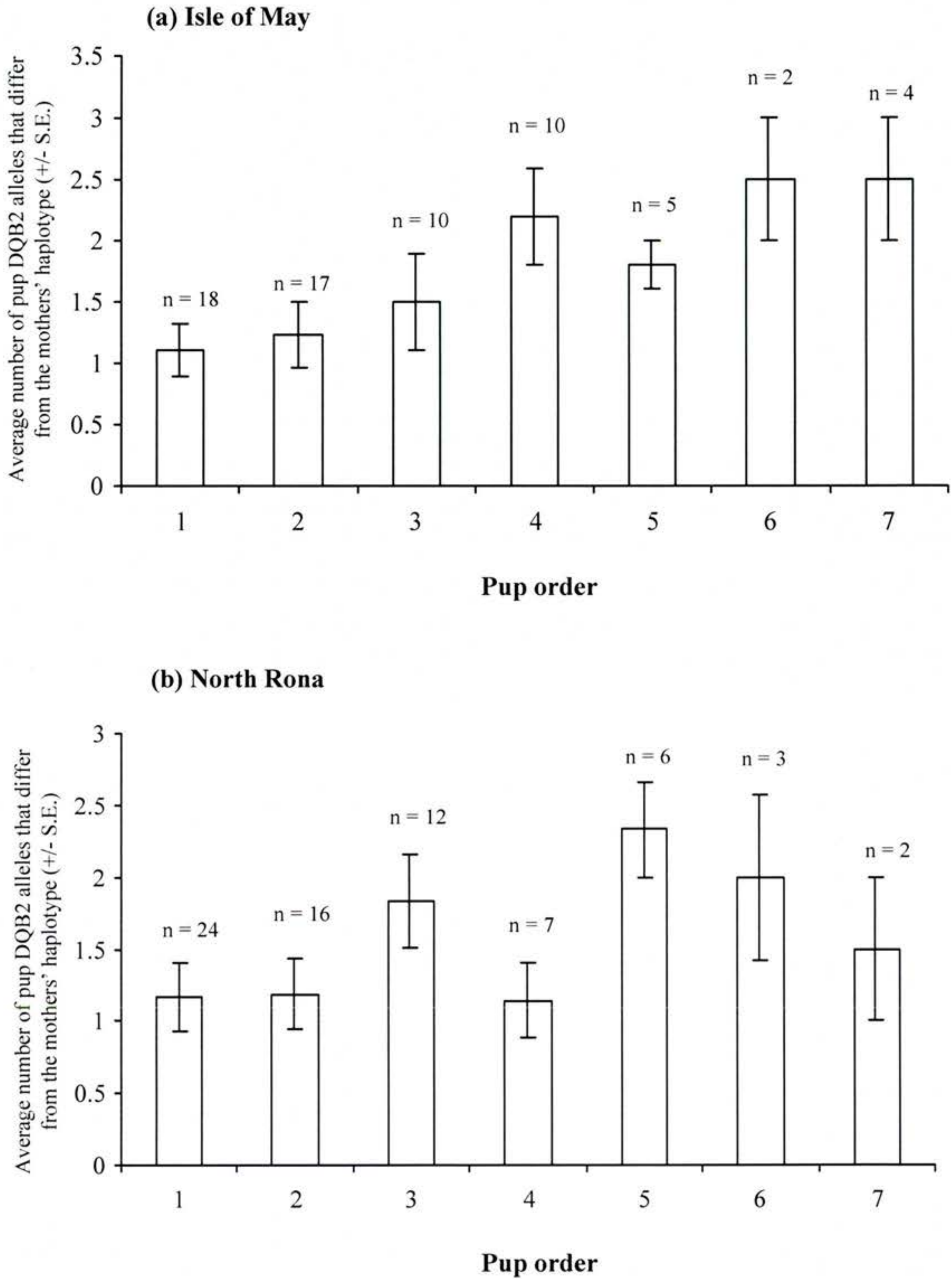

Figure 4.3 Average number of DQB2 allele differences (+/- S.E.) in pups in relation to the mothers' haplotype over consecutive pupping events from **(a)** the Isle of May and **(b)** North Rona.

4.3.2 Do females produce more heterozygous offspring than homozygous offspring?

Current theory in relation to MHC-correlated mate choice suggests that choice for MHC-dissimilar males will yield offspring that are maximally heterozygous. Hence, females will produce more heterozygous than homozygous offspring (Tregenza & Wedell 2000; Landry et al. 2001). Overall, on the Isle of May there were significantly more DQB2 heterozygous pups than homozygous pups (χ^2 = 7.65, d.f. = 1, P < 0.001 with Yates correction). However there were no significant differences in the proportion of heterozygotes and homozygotes over consecutive pupping events (χ^2 = 10.74, d.f. = 7, P > 0.05) (Figure 4.4).

In contrast, on North Rona there was no significant difference in the proportion of offspring that were homozygous and heterozygous at the DQB2 region (χ^2 = 0.48, d.f. = 1, P > 0.05 with Yates correction). In fact, on North Rona there were more homozygotes overall (n = 56) than heterozygotes (n = 48). Furthermore, there was no significant difference in the frequency of heterozygotes and homozygotes over consecutive pupping events (χ^2 = 2.56, d.f. = 7, P > 0.05) (Figure 4.4).

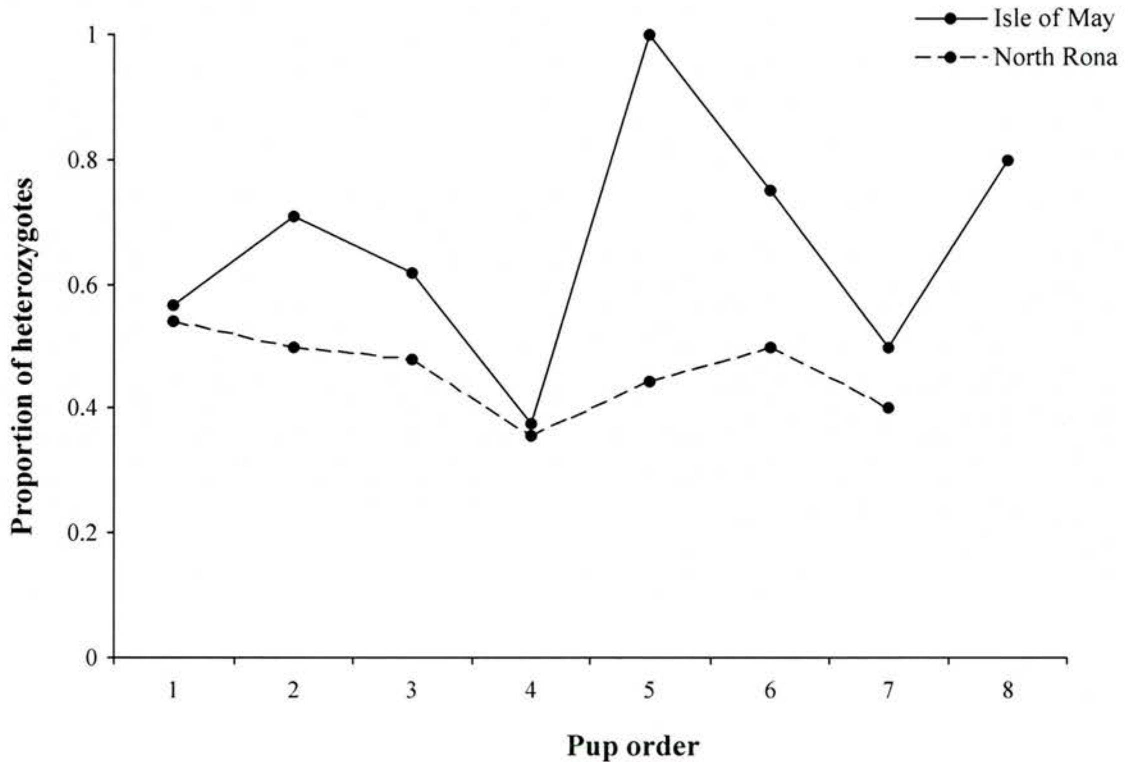

Figure 4.4 The proportion of grey seal pups that are heterozygous at the DQB2 region over consecutive pupping events on the Isle of May (1991 and 2002: n = 110) and North Rona (1985 and 1999: n = 104).

4.3.3 Variation in DQB2 diversity between mothers and pups

If females choose males with the aim of increasing levels of DQB2 diversity in their offspring, pups are expected to have higher overall levels of DQB2 diversity than mothers. On the Isle of May and North Rona there was no significant difference in DQB2 diversity between mothers and pups over consecutive pupping events (Isle of May: $F_{1,137} = 1.58$, $P > 0.05$, North Rona: $F_{1,129} = 0.89$, $P > 0.05$) (Figure 4.5). Interestingly, for the Isle of May the sixth, seventh and eight born pups had considerably lower levels of DQB2 diversity than previous pups and in relation to mothers (Figure 4.5).

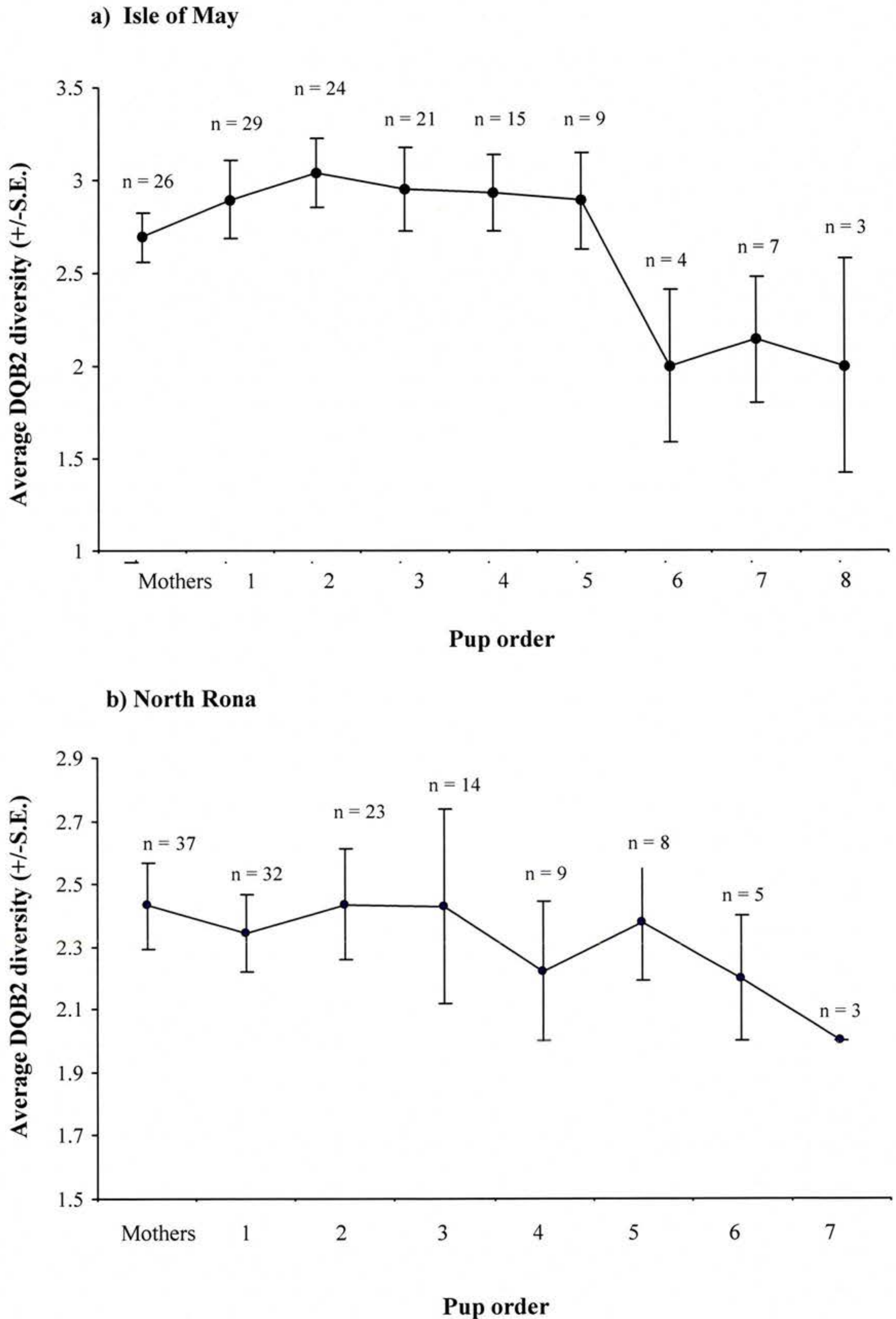

Figure 4.5 Average DQB2 diversity (+/- S.E.) for grey seal mothers and pups on the Isle of May (nine breeding seasons between 1991 to 2002) and North Rona (ten breeding seasons between 1985 to 1999). Sample sizes are indicated.

4.3.4 Proportion of DQB2 alleles in mothers and pups

If certain MHC alleles are important for fitness or confer increased protection from pathogens, females should choose males that carry those alleles. On the Isle of May there were significant differences in the proportion of alleles in mothers and pups summed over all years (χ^2 = 61.68, d.f. = 9, P < 0.001) (Figure 4.6). When the proportion of DQB2 alleles is compared over consecutive pupping events, there is a trend where alleles 2 and 4 become less common (locus L1) and alleles 1 and 5 (L3) increase in frequency (Figure 4.6)

On North Rona there were no significant differences in the proportion of DQB2 alleles found in pups from all years compared to mothers (χ^2 = 4.93, d.f. = 9, P > 0.05). However, examination of allele frequencies in pups over consecutive pupping events reveals fluctuations in the proportion of alleles (Figure 4.6). In particular, there was a considerable increase in allele 3 and a decline in allele 4 (Figure 4.6).

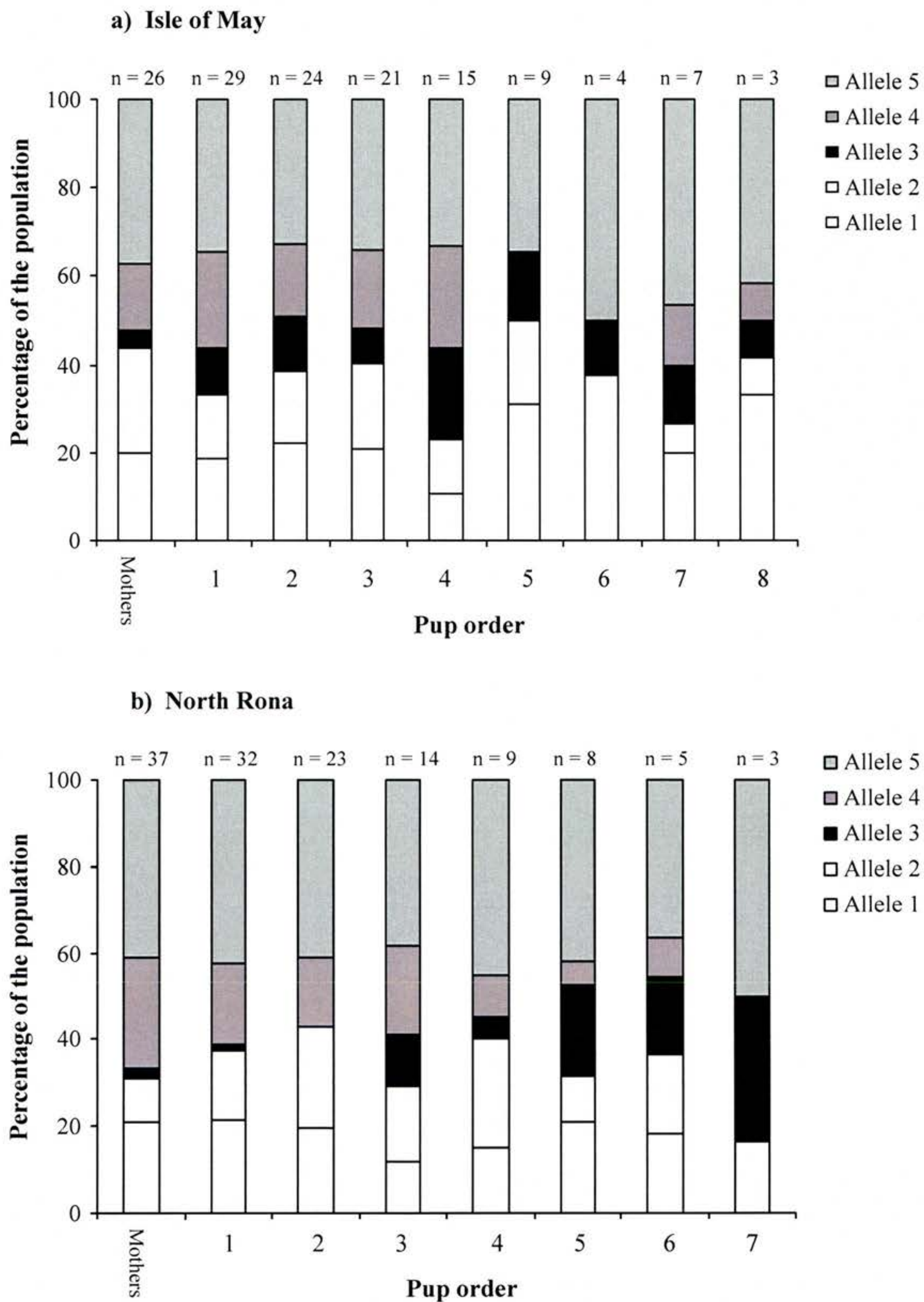

Figure 4.6 The percentage of each DQB2 allele found in all grey seal pups and mothers on **(a)** the Isle of May and **(b)** North Rona. Sample sizes are indicated above each bar.

4.4 Discussion

4.4.1 DQB2-dissimilarity between mothers and pups

One of the main findings of this study is that there are significant differences in the number of DQB2 alleles in pups that differ from the maternal haplotype between pupping events. Furthermore, there were considerable differences between the two islands in extent to which levels of DQB2-dissimilarity varied over consecutive pupping events. The variation in levels of DQB2-dissimilarity between the two UK islands observed here may reflect the proportion of individuals within each population participating in different mating strategies. The number of individuals participating in mate choice, polygyny and aquatic mating could be influenced by female 'experience', topography and/or the degree of site fidelity on each of the islands. These will be discussed in turn below.

Primiparous pups on the Isle of May carry few DQB2 alleles that differ from those carried by their mothers. However, subsequent young have a greater number of DQB2 alleles that differ from the female haplotype. This finding is interesting and to my knowledge has yet to be documented in any species of mammal. One possible explanation for the temporal pattern observed here is that, as time progresses, a female's 'experience' may be important in determining which mating strategy she uses. Initially, young females may be involved in a polygynous mating strategy because they cannot either physically, due to smaller body size (Bonner 1972), or behaviourally, females often return to the same breeding locations (Pomeroy et al. 1994), avoid mating with the dominant male. In polygynous systems, pups would not on average be expected to be particularly DQB2-dissimilar from their mother. As time progresses, a female may be able to exert more choice in the male she mates with due to her larger size or past mating experience/s. Hence, pups born in subsequent years may show increasing levels

of DQB2 variation from the female haplotype as females become increasingly able to choose males that are DQB2-dissimilar from themselves.

Although there are significant differences in the levels of DQB2-dissimilarity between pupping events on North Rona, the temporal pattern is not as clear as it was on the Isle of May. It is possible that on North Rona a females choice for DQB2-dissimilar males is opportunistic rather than increasing with female age. One possible reason for this is that females breeding on North Rona have much higher levels of site fidelity than on the Isle of May (Pomeroy et al. 1994). Hence, fluctuation in the arrival time of dominant males or the spatial distribution of males that are available in different years may alter the degree to which mate choice for DQB2-dissimilar males can occur. If the dominant males happen to be MHC-similar to the resident females and arrive before any MHC-dissimilar males, there is little scope for female choice where site fidelity is high. As well as stochasticity in male arrival and distribution, topography may also affect the extent to which choice for DQB2-dissimilar males can occur. On the Isle of May, the topography of the island is such that fewer males are able to maintain central positions within the colony in the way they can on North Rona (Worthington Wilmer et al. 1999). Where males cannot hold an adequate territory within the colony there is the potential for alternate mating strategies to occur, which in turn can affect the level of DQB2-similarity between females and their offspring.

Results from this study indicate that there are a number of factors influencing mating strategies in grey seals, such that analysis of a single genetic variable is inadequate in describing the conditions under which each strategy may occur. The complex interplay between a female's age, topography, male status and genetic composition of the population makes the work described in this chapter just one piece in a remarkably complicated jigsaw.

4.4.2 Does DQB2 heterozygosity, diversity and the proportion of DQB2 alleles vary between mothers and pups on the Isle of May and North Rona?

Females on the Isle of May overall produce significantly more heterozygous than homozygous offspring, but there was no significant temporal trend relating to the females' age. The significantly higher proportion of heterozygous offspring suggests that females may be choosing mates in order to increase heterozygosity in their resulting offspring. This is because heterozygous individuals may be at a fitness advantage by being able to recognize a wider range of pathogens (Jeffery & Bangham 2000; Hedrick 2002). However, it is expected that levels of heterozygosity in pups over consecutive pupping events should reflect the same pattern observed using DQB2-disimilarity. This is because choice for DQB2-dissimilar males is expected to also result in an increase in heterozygosity (Mays & Hill 2004). This finding suggests that if females do choose DQB2-dissimilar males that this does not automatically result in increased heterozygosity. Furthermore, overall higher levels of heterozygosity may reflect a higher frequency of heterozygous males and females within this population rather than choice for heterozygosity *per se*. In contrast, on North Rona there are no significant differences in the proportion of homozygous and heterozygous offspring. Indeed, there were slightly more homozygotes overall. This suggests that on North Rona there may be lower levels of DQB2 heterozygosity in the population as a whole and that if choice does occur this would be difficult to detect without the inclusion of behavioural observations and determination of male haplotypes.

On the Isle of May and North Rona there was no significant variation in DQB2 diversity in pups over consecutive pupping events. However, for the Isle of May there was a sharp decline in DQB2 diversity after a female had had her sixth pup. Low sample sizes for the sixth, seventh and eighth pupping event categories suggest that this

decline may be an artefact of low sample size and larger sample sizes are required to determine if this trend is correct. Despite a lack of a statistically significant difference, pups on both islands had higher overall DQB2 diversity than mothers. This finding is consistent with the hypothesis that if females actively choose mates, they should choose males that will increase DQB2 diversity in their offspring. However, the lack of a statistically significant difference between levels of DQB2 diversity in mothers and pups means that conclusions about this result are tentative. Although both islands yield similar results when comparing DQB2 diversity between mothers and pups, pups and mothers on the Isle of May are more DQB2 diverse than mothers and pups on North Rona. As is mentioned above this difference between the two UK populations may be due to the proportion of individuals within each population which are participating in different mating strategies. Although polygyny is the dominant mating strategy (Bonner 1972), mate choice and aquatic mating strategies may be occurring at a higher frequency on the Isle of May compared to North Rona. This may lead to greater variation between mothers and pups in the level of DQB2 diversity.

The ways in which DQB2 directly affects mate choice *per se* may be difficult to determine if alternative mating strategies mask the effect. It is rarely possible to directly observe the genetic consequences of different behaviours in large mammals, and in this particular system further behavioural research could thus be combined with genetic studies like this one. However, as in all such complex systems, it is unlikely that monocausal genetic determination of behaviour takes place. The true effects of DQB2 diversity on mate choice may be masked by other factors that affect females' behaviour, such as the number and 'quality' of available males.

Investigation of the proportion of particular alleles in mothers and pups suggests that, on the Isle of May, females may be selecting for particular alleles or that these

alleles are common in the dominant males. For example, both alleles from locus L1 decline as a female ages whereas alleles from locus L3 become progressively more common. Similarly, on North Rona allele 4 (from locus group L1) declines considerably over consecutive pupping events while allele 3 becomes increasingly more common. The increase in alleles 1, 5 and 3 may either reflect choice for males carrying those alleles or that males within these populations do not carry these alleles. Determination of male haplotypes will allow for the availability of different DQB2 alleles with each population to be calculated. The analyses of levels of DQB2 diversity and heterozygosity in males will aid in determining if pups have higher levels of diversity, heterozygosity and carry particular alleles in higher frequencies than would be expected from random mating. There is clearly the need to include male, female and pup genotypes together with behavioural observations and other demographic parameters within a multivariate statistical framework to provide a clearer understanding of how DQB2 variation may effect mating strategy in the grey seal.

4.4.3 Conclusions

Results from this chapter suggest that the importance of the DQB2 region on mating strategy varies between the two island populations. This variation may be due to the degree of alternative mating strategies operating within each colony where on the Isle of May mate choice may occur more frequently than on North Rona. On the Isle of May, high levels of MHC-dissimilarity between mothers and pups indicate that a female's past reproductive experience and/or age may affect the type of mating strategy she uses. In addition, females on the Isle of May produced significantly more DQB2 heterozygous than homozygotes offspring. This suggests that females on the Isle of May may choose DQB2-dissimilar mates in order to increase heterozygosity and in turn

fitness in their resulting offspring. On North Rona there were no clear patterns in relation to DQB2-dissimilarity, diversity and heterozygosity although there was considerable year-on-year variation in the proportion of particular alleles and a significant difference between levels of DQB2 dissimilarity in pups. Further studies which include behavioural observations of females and the males they mate with may allow each mating strategy; polygyny, mate choice and aquatic mating to be investigated in relation to variation at the DQB2 region in more detail. Inclusion of male genotypes in analyses investigating the mating behaviour in grey seals will be useful in providing an indication of levels of DQB2 diversity, heterozygosity and the frequency of particular alleles within the male population.

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CHAPTER 5

The relative importance of inbreeding and the DQB2 region in pre-weaning grey seal pup survival

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CHAPTER 5

The relative importance of inbreeding and the DQB2 region in pre-weaning grey seal pup survival

"It is a wise father who knows his own child". "It would be an even wiser child who knows her own father"
William Shakespeare, The Merchant of Venice

5.1 Introduction

In large mammals such as grey seals, the first few weeks or months of life often carry the most threats, both from predation and disease (Clutton-Brock et al. 1985; 1987; Hall et al. 2001). There are thought to be a number of factors that contribute to disease susceptibility and resistance. One kind of susceptibility is general and can result from an individual having poor body condition. This is because poor body condition at an early stage of life is often associated with sub-optimal immune response (Gershwin et al. 2000), where there is expected to be a trade off between energy devoted to growth and reproduction and that required for a strong immune system (Hall et al. 2002). Alternatively, genetic factors such as inbreeding depression, the loss of fitness that results from having two related individuals as parents, can also contribute to disease susceptibility (Coltman et al. 1998; Cassinello et al. 2001; Acevedo-Whitehouse et al. 2003). Inbreeding depression has been linked to reduced offspring survival in numerous species of birds (for examples see Brown & Brown 1998; Keller 1998; Daniels & Walters 2000) and mammals (for example see Coltman et al. 1998; Coulson et al. 1998). In addition, there may also be more specific effects attributable to particular genes. One of the best known genetic regions involved in fighting pathogens

is the MHC (Hughes et al. 1994; Potts et al. 1994; Penn & Potts 1999; Jeffery & Bangham 2000).

As yet, the exact nature of a 'good' MHC haplotype is unclear. It has been postulated that increased heterozygosity at the MHC region can enhance resistance to disease by increasing the diversity of antigens presented to T cells (Hedrick 2002; Penn et al. 2002; McClelland et al. 2003). For example, in humans MHC heterozygosity has been linked to prolonged survival of HIV-infected individuals and decreased susceptibility to malaria (Hill et al. 1991) and diabetes (Carrington et al. 1999). However, these findings may also reflect heterozygosity at other loci and not be causally linked to MHC heterozygosity. It has also been suggested that expression of rare alleles at the MHC may decrease the probability of infection and increase fitness due to an increased ability to fight novel pathogens (Jeffery & Bangham 2000; Langefors et al. 2000; Landry et al. 2001). This is commonly referred to as the 'rare allele hypothesis'. It is possible that particular alleles may also confer susceptibility to specific pathogens. For example, evidence for a positive association between particular MHC alleles and fitness has been found in laboratory studies on salmon (*Salmo salar*) (Langefors et al. 2000; Lohm et al. 2002). In these studies, the MHC class IIB allele *e* was significantly more prevalent in fish that displayed a greater resistance to infection by the bacterium *Aeromonas salmonicida* (Langefors et al. 2000; Lohm et al. 2002).

To date, most of the studies attempting to correlate MHC heterozygosity and the presence of particular MHC alleles with fitness have been focused on humans (Carrington et al. 1999) or have been based on laboratory studies of other species (Doherty & Zinkernagel 1975; Reusch et al. 2001). Similarly, studies on the relationship between offspring survival and levels of inbreeding have been based on captive or laboratory studies, although a few studies on wild populations have now been

published (see Slate et al. 2000; Acevedo-Whitehouse et al. 2003). However, extrapolation from such laboratory-based situations to wild populations is problematic, as controlled lab studies are rarely able to mimic conditions relevant to survival in the wild and may lead to unrealistic views of the factors that determine fitness and survival.

The grey seal is an ideal species in which to investigate the influence of inbreeding and MHC variation on survival in a wild population for two reasons. First, the time between birth and weaning in grey seals is particularly short for a large mammal, lasting approximately 18 days, after which pups are abruptly weaned. At this stage, pup mortality is high, thereby allowing dead pup skin samples to be collected from many individuals over a short period of time prior to weaning. Second, causes of mortality have long been studied in this species assisting diagnosis of cause of death in the field.

5.1.1 Causes of pre-weaning grey seal mortality

Factors that contribute to grey seal mortality during the pre-weaning period are numerous, but relatively few are responsible for the bulk of deaths (Baker 1987). For example, starvation alone is responsible for approximately 26 % of deaths (Baker 1988; Baker & Baker 1988) and primarily arises from a lack of bonding between mother and pup immediately after birth (Anderson et al. 1979). Because breeding beaches are extremely crowded, failure to establish a mother-pup bond immediately after birth will often result in the pup being abandoned by the mother. The pup then usually dies unless it is fostered by another female (McCulloch et al. 1999).

Crowded conditions on breeding beaches can cause death in other ways, in particular by traumatic incidents. Because animals are tightly packed and recognition between females and their pups can sometimes be inaccurate, pups sometimes attempt

to suckle from females that are not their mothers, and usually get bitten in response. This can lead to severe injuries and subsequent secondary infections. Pups can also be crushed during fights between females, or during mating attempts by resident males and females. Furthermore, when startled, adults often stampede towards the water, inadvertently crushing pups (Baker 1984). A further cause of death is drowning. Although pups can swim from birth and are commonly seen with their mothers in the water, pre-weaned pups are often found drowned (Anderson et al. 1979; Baker 1984; 1988; Baker & Baker 1988). This occurs as a result of storms, strong currents/waves and undertows, being washed off rocks and being entangled in objects (Baker 1984).

Crowded conditions within the colony can also lead to infection by any of several diseases, which is often exacerbated by unclean conditions (Anderson et al. 1979; Baker 1984). Pups may also be more susceptible to opportunistic infections than adults due to low immunoglobulin concentrations in their first five weeks of life (Carter et al. 1990). In nearly all studies investigating mortality in grey seal pups, peritonitis and 'other' infections are documented amongst the primary causes of death (Anderson et al. 1975; Baker 1984; 1988; Baker & Baker 1988). Reported infections include generalised bacterial infection and infection of the lungs, pleura and liver amongst other organs (Anderson et al. 1975). Of these infections, peritonitis is the most common and is due to *Streptococcus spp.* and *Corynebacterium spp.* (Anderson et al. 1979; Baker 1988). Infection of the peritoneum and 'other' infectious diseases are more likely in 'dirtier' environments, close to wallows and in gullies (Baker & Baker 1988). One of the other main causes of mortality is still birth which contributes a further 5 % to 15 % of deaths (Baker 1984). There is a host of other contributory causes of death such as heart abnormalities, but these are relatively rare compared to those listed above (Baker et al. 1980). Therefore, in pre-weaned grey seal pups approximately one third of deaths

are due to starvation and trauma, another third is due to peritonitis and other infections and the remainder is caused by a combination of rarer ailments (Baker et al. 1980).

5.1.2 Aims

Despite the extensive observational literature on factors contributing to pre-weaning pup mortality in grey seals (Anderson et al. 1979; Baker 1984; 1987; 1988; 1989; Baker & Baker 1988; Baker et al. 1998) none has yet investigated the extent to which levels of inbreeding, and in particular variation at the MHC, may contribute to pup mortality. Both these estimates of genetic variation have the potential to play an important role in survival of young. In this chapter, I investigate the relationship between survival of pre-weaned pups from three different colonies (Isle of May, North Rona and Sable Island), in relation to inbreeding (estimated as internal relatedness (IR) and standardised mean d^2 (S mean d^2)) and variation in the DQB2 region (measured as DQB2 diversity, heterozygosity and the proportion of each allele/haplotype). The relationship between the DQB2 region and inbreeding with possible cause of mortality and pup stage were also investigated for dead pups on the Isle of May.

5.2 Materials and methods

5.2.1 Live pup sample collection

Skin samples from live pups on the Isle of May and North Rona comprise those taken from pups of the focal females described in chapter four (Isle of May n = 214, North Rona n = 284) (for full details of DQB2 and microsatellite genotyped samples see chapter two). Skin samples from grey seal pups on Sable Island (n = 28) were collected by Prof Ian Boyd (SMRU, St Andrews University) in 2003 only. Each pup was restrained in a large bag and the weight to the nearest gram (using a hand held balance scale) and sex were recorded. Further to this, since accurate birth dates are not usually available and pup stage is known to be a good proxy for age, each pup was also staged using a five-point scale based on pelage colour, body size and development characteristics (see Table 5.1 and Figure 5.1 for examples of stage 2 and 5 pups) (Kovacs & Lavigne 1986).

5.2.2 Dead pup sample collection

Skin samples were collected opportunistically from dead pups by previous researchers on North Rona between 1986 and 2002 (n = 87) and the Isle of May between 1991 and 1996 (n = 158). I collected additional samples on the Isle of May in 2002 (n = 144). Skin samples from the Isle of May were provided by Simon Moss in 2003 (n = 109), and from Sable Island by Prof Ian Boyd in 2003 (n = 40). For full details of DQB2 and microsatellite genotyped samples see Table 2.1. Skin samples were collected using pig-ear notching pliers to remove a small piece of skin from the interdigital webbing of a hind flipper. All skin samples were stored in DMSO saturated with salt (Amos & Hoelzel 1991), except for those collected in 2002 and 2003 which were stored in 95 % ethanol.

a)

b)

Figure 5.1 a) A stage two 'white coat' pup and **b)** a fully weaned grey seal pup that has moulted the white coat to reveal the adult pelt (stage 5 pup).

5.2.3 Pup necropsies

As well as examining if levels of inbreeding and variation at the DQB2 region differ between live and dead pups from different islands, these measures were used to examine possible cause of mortality for dead pups on the Isle of May. In the 2002 breeding season, a systematic scheme was implemented on the Isle of May in which as many dead pups as possible were necropsied. For this year only, possible cause of mortality was determined (see Table 5.2). Approximate time of death was estimated using standard classifications (Table 5.1). Necropsies included both external and internal examinations and were conducted on all pups found less than approximately 24 hours post-mortem (PM) (n = 76). Those that had died more than 24 hours before examination took place were assessed for stage, examined externally for signs of lesions, given a post-mortem indicator value (PMI, Table 5.1), and assessed for blubber thickness and quality (see section 5.2.3.1 and Table 5.1 for full description of how this was determined). Pups that were classified as malnourished were not staged as these individuals tend to moult early, taking on the coat characteristics of a later stage pup and could therefore not be accurately classified into a particular stage.

Table 5.1 Classification of blubber quality, carcass condition (post-mortem indicator) and pup stage.

Category	Classification	Characteristics
Blubber quality	1	White
	2	Pink/white
	3	Pink
	4	Pink/red
	5	Red
	6	Yellow or discoloured
Carcass condition PMI (Post-mortem indicator)	<12 hours	No bloating, still in rigor mortis, eyes usually still intact, hair does not pull out easily.
	<24 hours	Tail flippers may still be in rigor mortis, hair does not pull out easily, no bloating.
	>24 hours	Body limp, eyes missing, hair pulls out easily
	>36 hours	Skin missing, mucus membranes are dry, hair pulls out easily, skin may also come off, bloated and dry.
Pup stage (adapted from Kovacs & Lavigne 1986)	1	New born, yellow–white coat, skin is limp and loose, ribs visible can be very small in size. In live pups movements are poorly coordinated and weak.
	2	White coat with no yellow patches, small, rounded body, ribs not visible.
	3	Thick white coat. Barrel-like body.
	4	Moulting around head and flippers.
	5	Fully moulted and no longer with mum. ***This stage was classified as a fully weaned pup.***

5.2.3.1 Internal examination of dead pups

Determination of the factors contributing to death and the subsequent identification of the main cause of mortality in an individual requires detailed diagnostic information (Gulland 1997). This usually includes the collection of tissue and blood samples for pathological analysis such as serology and histology as well as the examination of gross anatomical features. Due to logistic limitations during work in the field a complete diagnostic ascertainment of dead pups was not possible. Instead, I used a series of physical signs to broadly assign a cause of death to the best of my knowledge (see below and Table 5.2).

In order to examine dead pups internally, a longitudinal incision was made along the pups' ventral surface from the symphysis of the mandible to the anus or vulva.

Firstly, blubber thickness was measured to provide an indication of body condition and to determine if the pup was malnourished. Blubber thickness was measured at the sternum between the front flippers to the nearest millimetre using a ruler. In addition, pup condition and nutritional status were assessed qualitatively by blubber depth and characteristics (see Table 5.1) (Barnett et al. 2000; Rowles et al. 2001). Under stress and/or malnutrition, blubber thickness is often reduced and blubber colour can change from white to red (Rowles et al. 2001). Presence of milk in the stomach or mouth was ascertained to assist in determining if a pup may have been starved-out or still born. In order to aid in determining if a pup may have drowned or been born dead, a small 2 x 2 section of lung was placed in water. If the lung tissue sank this indicated that the pup had either not taken any air into the lungs (still born) or that the pup had inhaled water (drowned). Presence of the umbilical cord was a further indication of still birth in pups that showed no signs of having inhaled water. Where present, peritoneal fluid was categorised by colour; red, straw or clear in colour, to provide an indication of health status i e. red suggested an infection and straw/clear colour indicated protein leakage, probably as a result of malnutrition. Lastly, the appearance of the adrenal glands was used as a measure of stress. Where the cortex was enlarged and deviated from a medulla/cortex ratio of 3:1 the glands were classified as active, indicating that the individual was under stress (St Aubin 2001). Taking all PM observations into account, individuals could be placed into one of five 'possible cause of mortality' categories based on the main factor that appeared to contribute to death (Table 5.2). Where there was insufficient information to assign a most-likely cause of mortality, individuals were excluded from analyses involving this information.

Table 5.2 The main categorical cause, criteria used and the probable factors contributing to death for grey seal pups on the Isle of May 2002.

Possible cause of mortality	Criteria	Possible causal factor
Still born	Lung tissue sinks in water, umbilicus may still be attached.	Unknown
Infection	Icteric blubber, major pathognomonic lesions in other organs. Presence of peritoneal fluid that was red or straw coloured with red flecks. Fibrinous adherences to peritoneum or abdominal wall.	Leptospirosis, brucellosis, Hepatitis.
Trauma	Bite marks, broken limbs and ribs in the absence of, or very limited signs of, malnutrition.	Attack from adult seals, falls, abrasion against rocks.
Malnutrition	Nearly no body fat but when present fat is pink to red in colour. Peritoneal fluid is clear or straw coloured.	Abandoned pup or pup separated from mother.

5.2.4 Data analysis

See section 2.4.4 (chapter two) for detailed descriptions of how the two estimates of inbreeding used in this study (IR and S mean d^2) are calculated. To determine whether the two estimates of inbreeding and DQB2 diversity (number of alleles carried by an individual) varied between the years in which samples were collected and/or with the sex of a pup, a generalised linear model (GLM) was constructed with a normal error structure. Analyses of whether measures of inbreeding varied with factors such as if the pup was found alive or dead, cause of death, colony of sampling or pup stage were conducted using one and two-way ANOVAs. Analysis of S mean d^2 in relation to pup stage was conducted using a Kruskal-Wallis test for K independent samples due to the inability to stabilise variances (Sokal & Rohlf 1995).

In order to determine if DQB2 diversity varies between islands and if a pup was live or dead a two-way ANOVA was conducted. Variances were heterogeneous and data were therefore $\log_{10}(x+1)$ transformed to stabilise variances (Sokal & Rohlf 1995).

The proportions of homozygotes and heterozygotes on each island were compared using chi-squared analysis with Yates correction. Comparison of the frequency of haplotypes in live and dead pups pooled over all islands was also analysed using chi-squared. However, chi-squared was only conducted for comparisons that had expected values greater than five. Yates' correction for two categories of data (one degree of freedom) and a Bonferroni correction for multiple tests were performed (Sokal & Rohlf 1995).

For dead pups on the Isle of May in 2002 for which a categorical cause of mortality could be assigned, a one-way ANOVA was conducted to investigate if DQB2 diversity varied in relation to possible cause of mortality. DQB2 diversity in dead pups and the stage at which they died was also analysed using a one-way ANOVA. The proportion of each allele in relation to whether a pup was live or dead and the extent to which the proportion of each allele varied between island populations and in relation to different causes of mortality was examined graphically.

5.3 Results

5.3.1 Cause of mortality assignment

Of the 124 grey seals examined in 2002, 85 were assigned to a possible cause of mortality category (Table 5.3). Thirty-nine individuals could not be placed into a cause of mortality category and were not used in subsequent analyses. These individuals were grouped as 'unknown'.

Table 5.3 Number of grey seals assigned to each possible cause of mortality category.

Possible cause of mortality	Number of individuals
Malnutrition	37
Still born	14
Trauma	14
Infection	19
Unknown	39
Total	124

5.3.2 Variation in the level of inbreeding between live and dead pups

Neither a pup's sex nor the year of sampling revealed a significant relationship with either estimate of inbreeding, or with DQB2 diversity (Table 5.4). Dead pups had significantly higher IR values than live pups (two-way ANOVA: $F_{1,1082} = 4.935$, $P < 0.05$) (Table 5.5). These differences were significant for IR in the UK populations but not at Sable Island (Figure 5.2). The non-significant differences at Sable Island might be due either to a genuine lack of difference, or to the smaller sample size.

S mean d^2 showed no overall difference between live and dead pups but did differ significantly between islands (two-way ANOVA: $F_{2,1082} = 21.906$, $P < 0.001$) (Table 5.5). In addition, dead pups on Sable Island had significantly lower S mean d^2

compared with live pups (two-way ANOVA: $F_{2,1082} = 3.388$, $P < 0.05$), suggesting that in this population inbred pups are less likely to survive (Table 5.5). On the Isle of May and North Rona, there was no significant difference in S mean d^2 between live and dead pups (Figure 5.2).

Table 5.4 Generalised linear model of sex and the year in which pups were sampled in relation to internal relatedness (IR), standardised mean d^2 (S mean d^2) and DQB2 diversity (DQB2).

Measure	Factors	Type III sums of squares	d.f.	Mean square	F	*P*
Sex	IR	0.096	1	0.096	2.640	0.106
	S mean d^2	0.003	1	0.003	0.794	0.374
	DQB2	0.016	1	0.016	0.201	0.655
Year	IR	0.079	4	0.020	0.542	0.705
	S mean d^2	0.048	4	0.017	0.912	0.092
	DQB2	0.106	4	0.026	0.337	0.853
Sex * Year	IR	0.040	3	0.013	0.365	0.778
	S mean d^2	0.091	3	0.003	0.711	0.546
	DQB2	0.315	3	0.105	1.300	0.275
Error	IR	7.740	213	0.036		
	S mean d^2	0.905	213	0.004		
	DQB2	17.221	213	0.081		

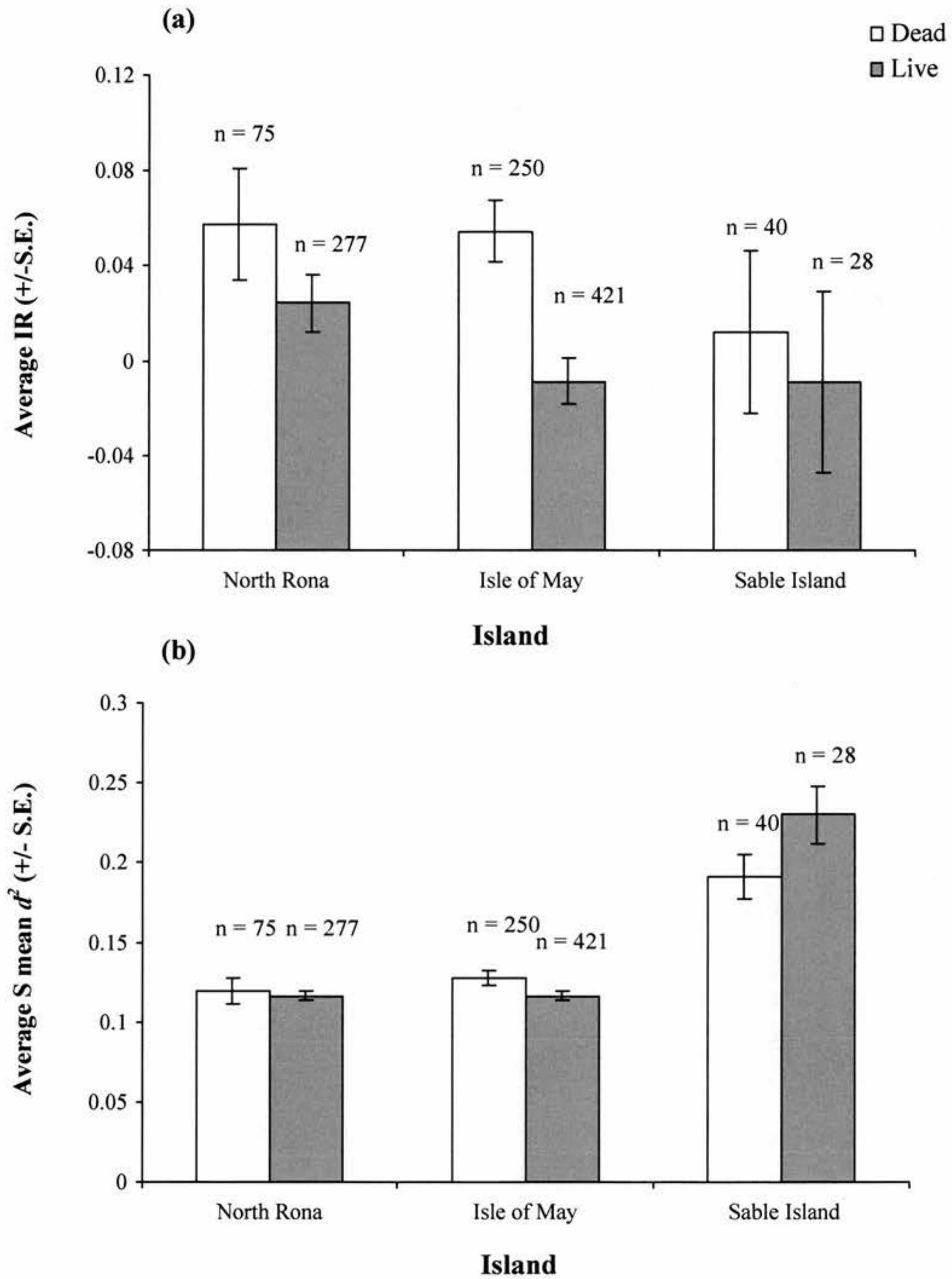

Figure 5.2 **(a)** Average internal relatedness (IR) and **(b)** average standardised mean d^2 (S mean d^2) (+/- S.E.) of live and dead pups on North Rona, the Isle of May and Sable Island. Sample sizes are indicated above each bar.

Table 5.5 Two-way ANOVAs analysing the relationship between internal relatedness (IR) and standardised mean d^2 (S mean d^2) of live and dead pups from three different island populations, North Rona, the Isle of May and Sable Island.

Inbreeding measure	Factors	Type III sums of squares	d.f.	Mean square	F	*P*
IR	Island	0.086	2	0.043	1.155	0.315
	Live vs dead	0.183	1	0.183	4.935	**0.027**
	Live vs dead * Island	0.042	2	0.021	0.561	0.571
	Error	40.054	836	0.037		
S mean d^2	Island	16.769	2	8.385	21.906	**0.000**
	Live vs dead	1.089	1	1.089	2.845	0.092
	Live vs dead * Island	2.593	2	1.297	3.388	**0.034**
	Error	414.130	836	0.383		

5.3.3 DQB2 variation in live and dead pups

Comparison of DQB2 allele diversity in live and dead pups from each of the three islands revealed that overall dead pups had significantly lower levels of diversity than live pups (two-way ANOVA: $F_{1,639} = 22.19$, $P < 0.01$) and that these differences were significant between islands (two-way ANOVA: $F_{2,629} = 5.432$, $P < 0.01$) (Table 5.6; Figure 5.3). The difference in mean allele diversity between live and dead pups was greatest at Sable Island, while on North Rona there was no difference in allele diversity between live and dead pups (Figure 5.3). Live pups at Sable Island also had the highest levels of allele diversity (mean DQB2 diversity = 3.50, S.E. = 0.209), whilst North Rona had the lowest (mean DQB2 diversity = 2.38, S.E. = 0.071) (Figure 5.3). There were no significant differences in DQB2 diversity in dead pups between each of the islands; North Rona (mean DQB2 diversity = 2.37, S.E. = 0.101), the Isle of May (mean DQB2 diversity = 2.29, S.E. = 0.057) and Sable Island (mean DQB2 diversity = 2.44, S.E. = 0.164) (Figure 5.3).

Table 5.6 Results of two-way ANOVA of DQB2 diversity of live and dead pups within three different island populations; the Isle of May, North Rona and Sable Island.

Factor	Type III Sums of squares	d.f	Mean square	F	*P*
Live/dead	1.548	1	1.548	22.190	**0.000**
Island	0.758	2	0.379	5.432	**0.005**
Live/dead * Island	0.904	2	0.542	6.482	**0.002**
Error	44.563	639			

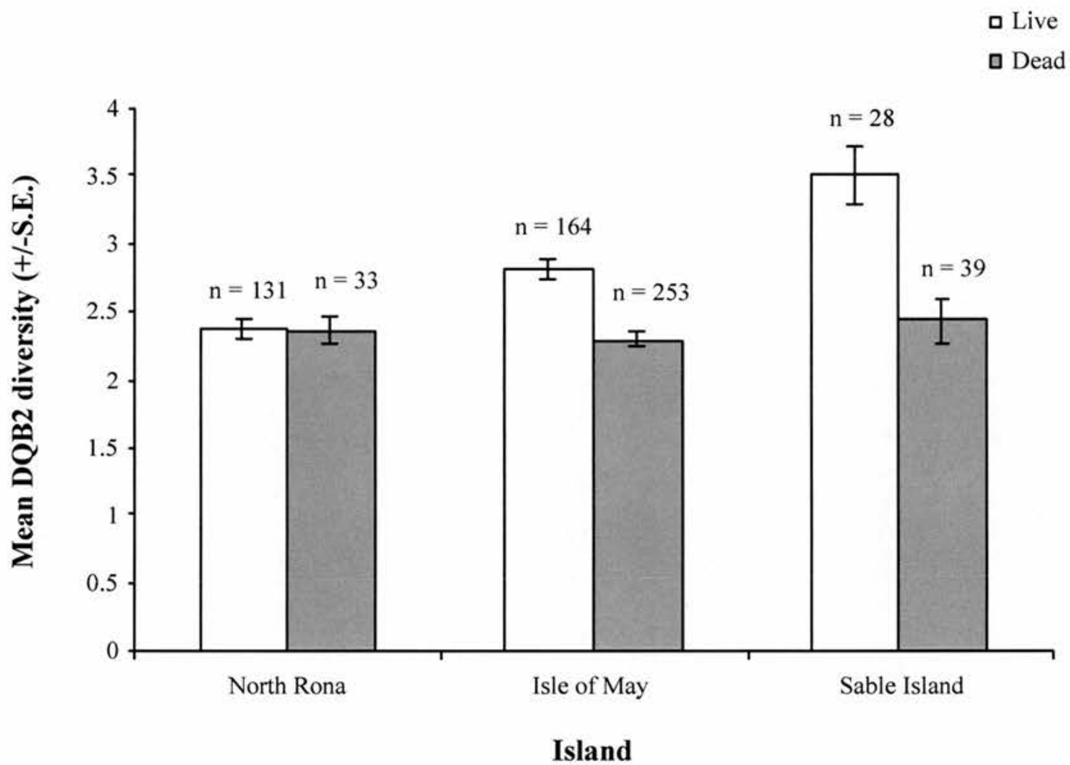

Figure 5.3 Mean DQB2 diversity (+/- S.E.) of live and dead pups at three islands; North Rona, the Isle of May and Sable Island. Sample sizes are indicated above each bar.

To determine the extent to which DQB2 homozygosity affects pup survival, the proportions of homozygotes and heterozygotes amongst live and dead pups from each population were compared using chi-squared analysis. On the Isle of May and Sable Island, there were significantly more homozygotes than heterozygotes amongst the dead pups (Isle of May = $\chi^2 = 44.29$, d.f. = 1, $P < 0.001$; Sable Island = $\chi^2 = 8.84$, d.f. = 1, $P < 0.005$ with Yates correction) (Figure 5.4). On North Rona, however, this was not the case. In this population, there was no significant difference in the proportion of homozygotes and heterozygotes in live and dead pups ($\chi^2 = 0.14$, d.f. = 1, $P > 0.05$ with Yates correction). As was the case for DQB2 diversity, Sable Island displayed the most marked difference in the proportion of heterozygotes in live and dead pups (Figure 5.4). Homozygous individuals made up 76.92 % of all dead pups sampled on Sable Island, but only 28.58 % of all live pups (Figure 5.4).

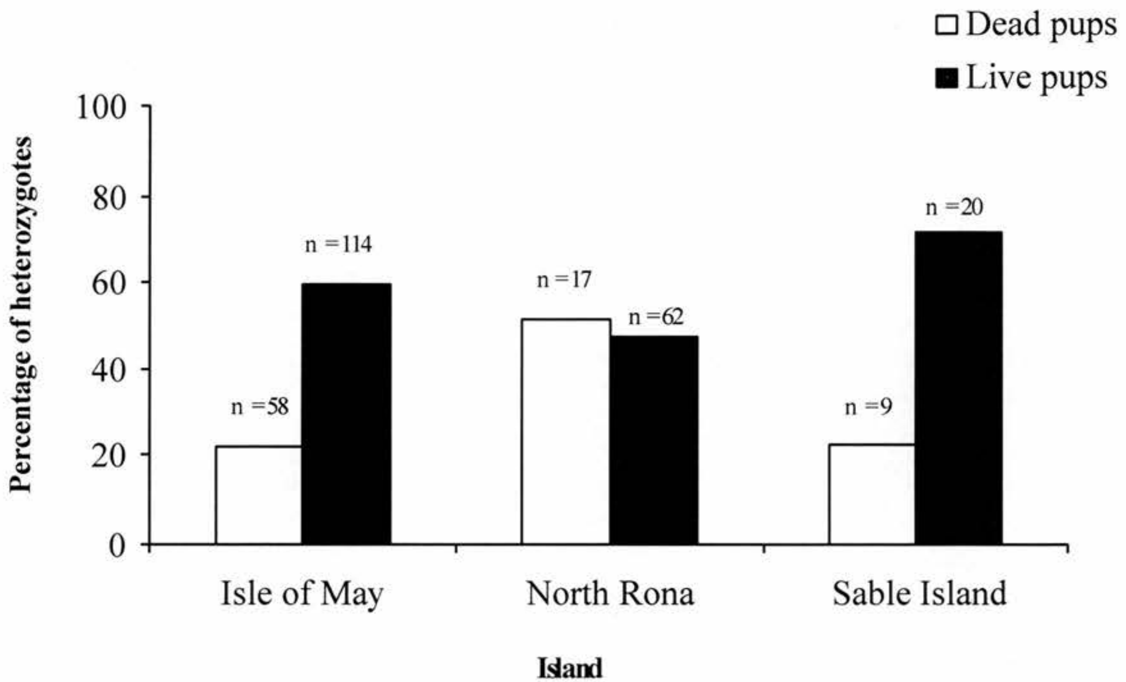

Figure 5.4 The percentage of heterozygotes in live and dead pups from three island populations, the Isle of May, North Rona and Sable Island. Sample sizes are indicated above each bar.

Significant differences exist between the haplotype frequencies found in live and dead pups (Table 5.7; Figure 5.5). Of the haplotypes analysed, haplotypes 3, and 3,4,5 were significantly more prevalent in dead pups than live pups (results of chi-squared analysis: χ^2 = 15.28, d.f. = 1, P < 0.001 and χ^2 = 20.76, d.f. = 1, P < 0.001 respectively with Yates and Bonferroni correction) (Figure 5.5). In contrast, haplotype 1,4,5 was significantly more prevalent in live pups than in dead pups (χ^2 = 13.03, d.f. = 1, P < 0.001 with Yates and Bonferroni correction) (Figure 5.5).

Table 5.7 Results from chi-squared analysis between the frequency of haplotypes in live and dead pups from all islands pooled. Chi-squared analysis was only conducted for comparison where the expected value was greater than 5. Yates correction for two categories of data and Bonferroni correction for multiple tests were performed. *** = $P < 0.001$

Haplotype	Chi-squared value
1,2,3,4,5	0.42
1,3,4,5	6.69
1,2,4,5	4.40
3	**15.28** ***
1,4,5	**13.03** ***
1,5	6.16
1,3,5	1.63
1,2,5	2.19
3,5	0.01
5	1.46
2,5	0.97
3,4,5	**20.76** ***
4,5	1.78

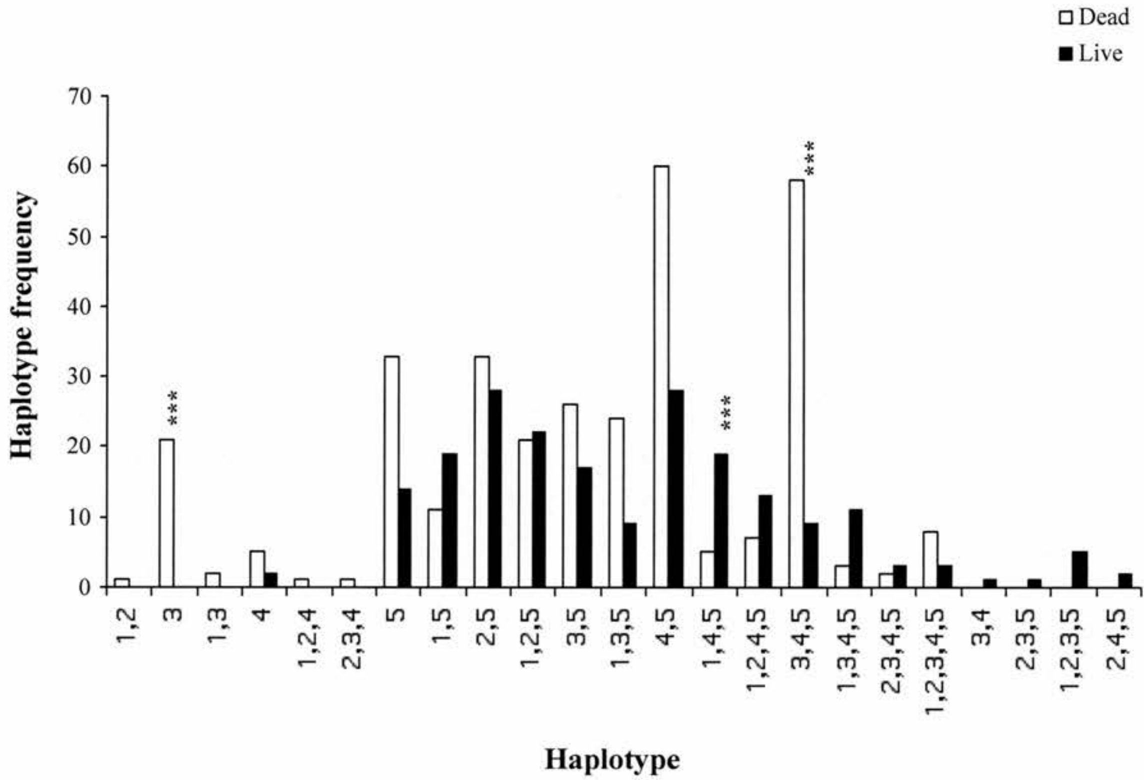

Figure 5.5 Frequency of haplotypes from live and dead pups pooled over all islands. Haplotypes are coded for by the alleles they contain. Significant differences between the frequency of a particular haplotype in live and dead pups are indicated, where *** = $P < 0.001$.

5.3.4 The effect of inbreeding and the DQB2 region on the possible cause of mortality of pre-weaned pups

My results suggest that inbreeding and DQB2 diversity both affect pup survival. However, it is also interesting to ask whether these measures also vary in relation to possible cause of mortality. There were no significant differences in the level of inbreeding using either measure and cause of mortality (Table 5.8). Although non-significant, there was a general trend where pups dying due to causes such as still birth and infection had slightly higher inbreeding values than those in the categories trauma and malnutrition (Figure 5.6). S mean d^2 displayed no clear trend with infection having the highest value (mean S mean d^2 = 0.15, S.E. = 0.02) and trauma the lowest (mean S mean d^2 = 0.098. S.E. = 0.01) (Figure 5.6).

Table 5.8 One-way ANOVAs of cause of mortality and level of inbreeding estimated as internal relatedness (IR) and standardised mean d^2 (S mean d^2) in dead pups from the Isle of May.

Inbreeding measure	Factors	Type III Sums of squares	d.f.	Mean square	F	*P*
IR	Cause	0.253	3	0.084	2.080	0.109
	Error	3.288	81	0.040		
S mean d^2	Cause	0.038	3	0.013	2.079	0.109
	Error	0.488	81	0.006		

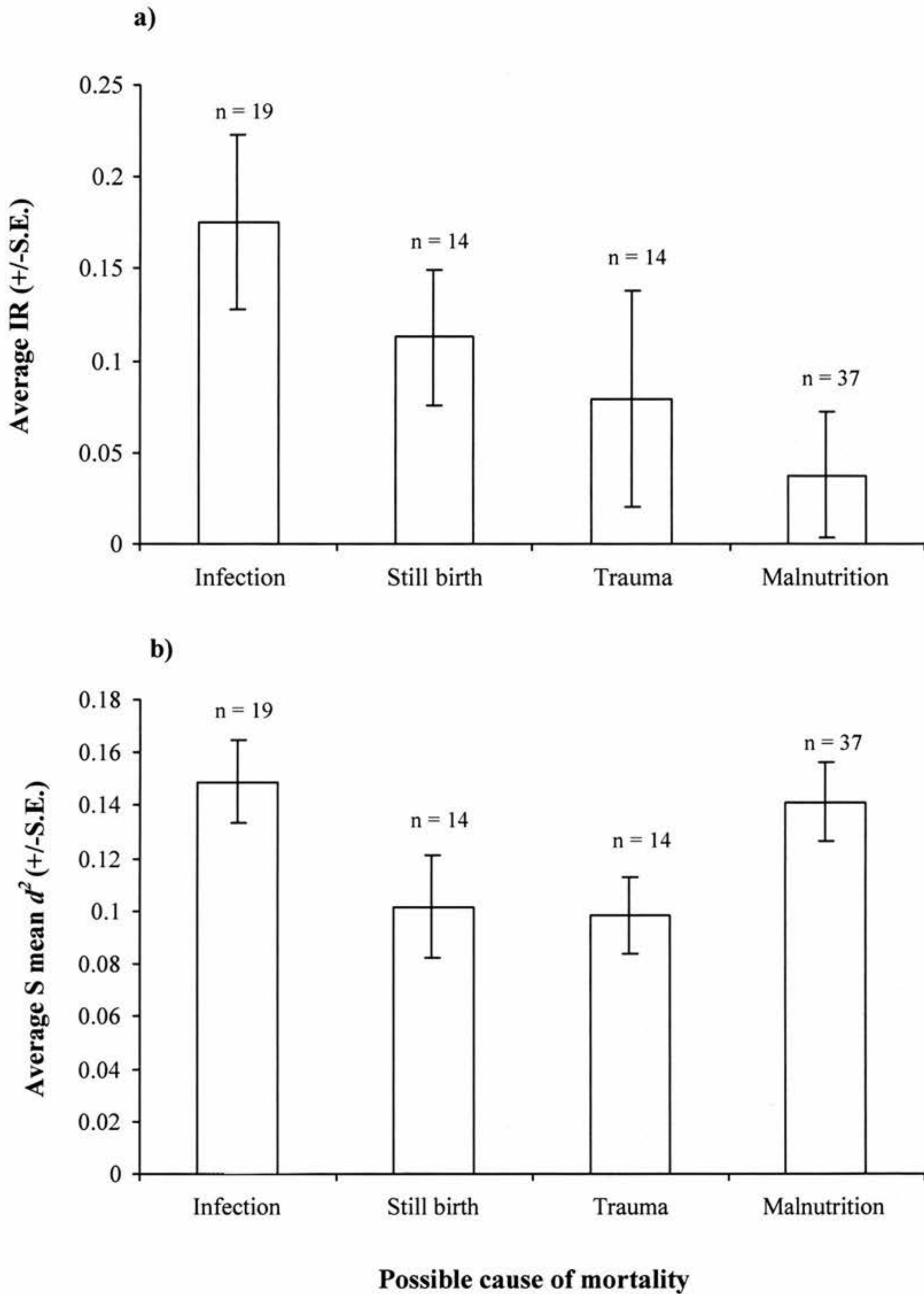

Figure 5.6 **(a)** Average internal relatedness (IR) and **(b)** average standardised mean d^2 (S mean d^2) (+/-S.E.) of pups which had died due to a variety of conditions; still birth, infection, trauma and malnutrition on the Isle of May 2002. Sample sizes are indicated above each bar.

There is no significant association between any of the five categorical causes of mortality and DQB2 diversity (results of Kruskal Wallis test for K independent samples expressed as: χ^2 = 3.6, d.f. = 3, P = 0.54) (Figure 5.7). Pups that were classified as belonging to the 'infection' category had the lowest level of DQB2 diversity with a large majority of individuals in this category carrying only two alleles (2 and 5) in the same combination (61.11 % of individuals carried this haplotype) (Figure 5.8). This differed from pups in the other four categories, where between 0 to 10 % of individuals carried the 2,5 haplotype (Figure 5.8). Pups that died from malnutrition had the next lowest level of DQB2 diversity (mean = 2.217, S.E. = 0.17), followed by pups that died of trauma (mean = 2.4, S.E. = 0.22) and those that were still born (mean = 2.5, S.E. = 0.42) (Figure 5.7).

Haplotype 2,5 was found to be particularly prevalent in pups classified as 'dead from infection' on the Isle of May, and as such a chi-squared analysis of the frequency of live and dead pups carrying this haplotype in each population was made. On Sable Island and North Rona, there were significantly more dead pups carrying the 2,5 haplotype than live pups with this particular haplotype (Sable Island: χ^2 = 6.0, n = 1, $P < 0.05$; North Rona: χ^2 = 4.19, n = 1, $P < 0.05$ with Yates correction) (Figure 5.9). On the Isle of May there was no significant difference in the number of live and dead pups carrying the 2,5 haplotype (χ^2 = 0.28, n = 1, $P > 0.05$ with Yates correction) (Figure 5.9).

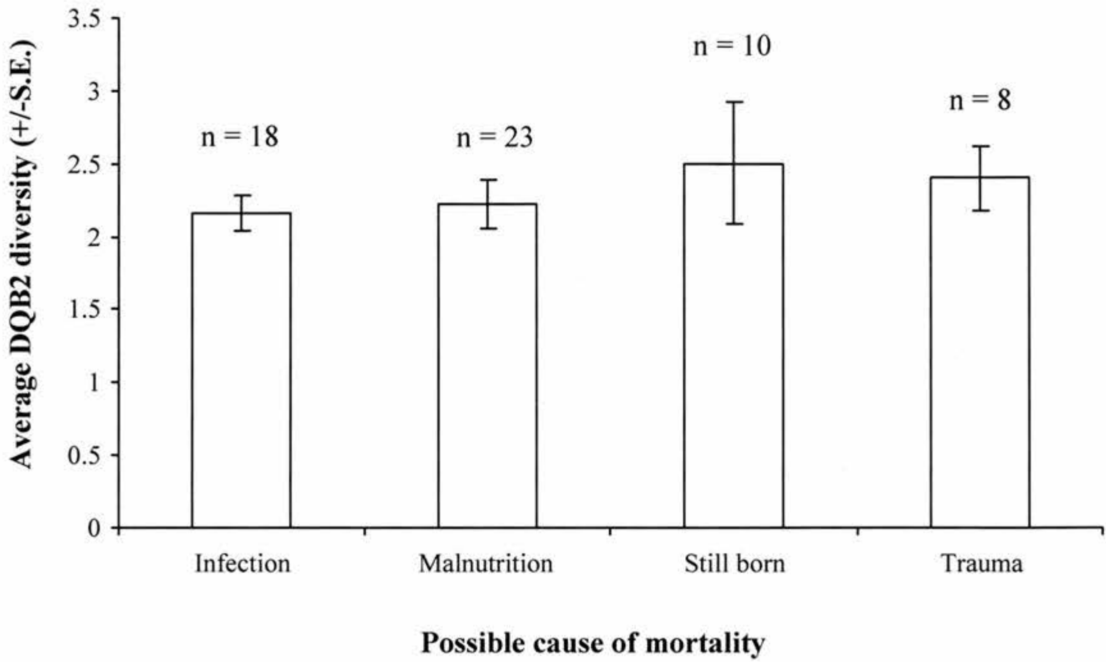

Figure 5.7 Average DQB2 diversity (+/- S.E.) in dead grey seal pups which had died due to infection, malnutrition, trauma and still birth on the Isle of May in 2002. Sample size for each of the categorical causes of mortality is indicated.

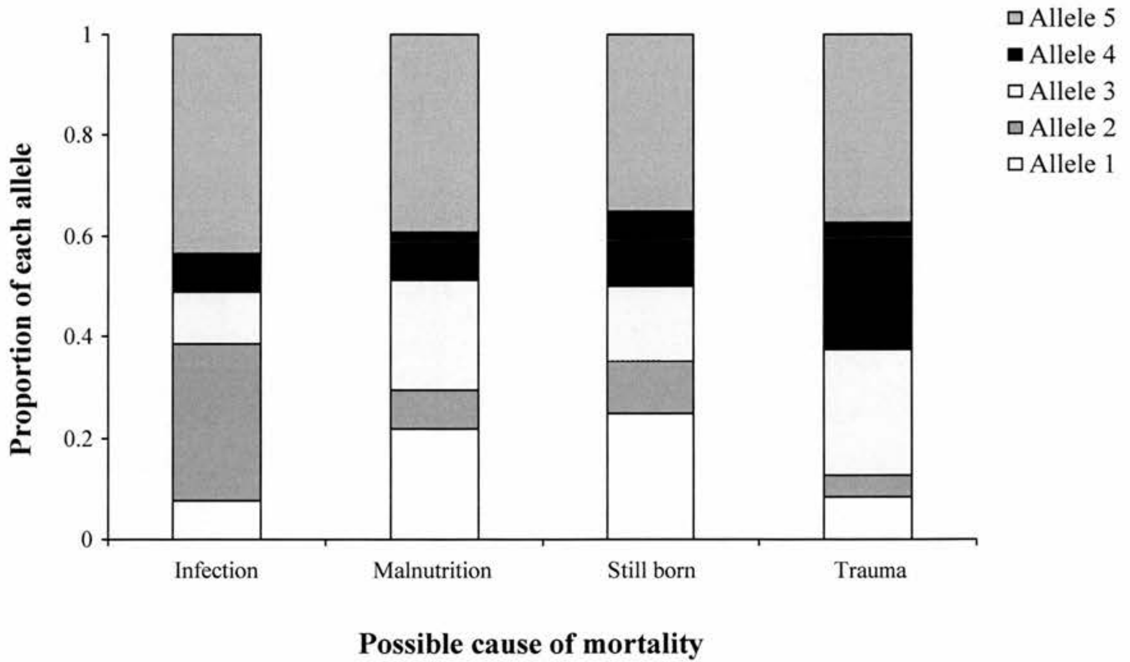

Figure 5.8 The proportion of each DQB2 allele from grey seal pups that had died due to infection, malnutrition, trauma and those that were still born on the Isle of May 2002.

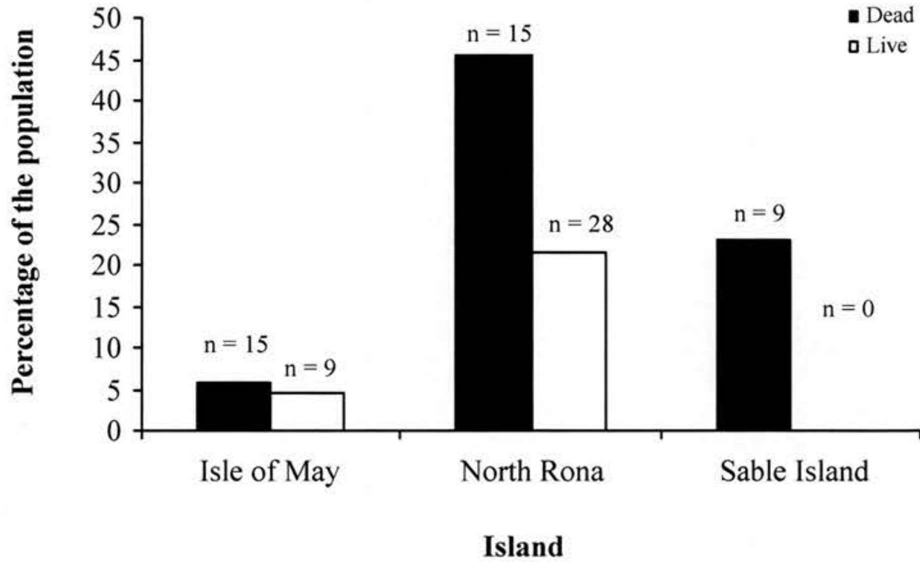

Figure 5.9 Percentage of live and dead pups within each island population that carry the DQB2 haplotype 2,5. Sample sizes are indicated above each bar.

5.3.5 Does inbreeding or DQB2 diversity vary with age of a pup at the point of death?

If levels of inbreeding and DQB2 variation influence offspring survival than it could be expected that dead stage one pups may have higher levels of inbreeding and lower DQB2 diversity. This is because the period around and immediately following birth is potentially a very traumatic period where genetic weaknesses may manifest most strongly. I used pup stage, which is a good indication of pre-weaning pup age, to investigate how inbreeding and DQB2 diversity vary with the age of a pup at the point of death. Although comparison of IR of dead pups and the stage at which they died revealed no significant difference between the stages (one-way ANOVA: $F_{4,129} = 0.906$, $P > 0.05$) (Table 5.9), there is variation in IR of dead pups amongst the stage classes

(Figure 5.10). For example, there is a considerable peak in IR at stage 4 just prior to weaning (mean IR = 0.24, S.E. = 0.05) (Figure 5.10). Pups that had died post-weaning (stage 5), had IR levels similar to that of live pups (mean IR = 0.11, S.E. = 0.01). There was also no significant association between S mean d^2 and pup stage (results of a Kruskal Wallis test for K independent samples expressed as: $\chi^2 = 3.794$, d.f. = 4, $P > 0.05$). Similarly, there was no significant relationship between DQB2 allele diversity and pup stage (one-way ANOVA: $F_{4,89} = 0.331$, $P > 0.05$) (Table 5.9; Figure 5.11).

Table 5.9 Results from one-way ANOVAs of the relationship between internal relatedness (IR) and DQB2 diversity (DQB2) and the stage at which a pup died.

Inbreeding measure	Factor	Type III Sums of squares	d.f.	Mean square	F	*P*
IR	Stage	0.037	4	0.037	0.906	0.343
	Error	6.017	129	0.040		
DQB2	Stage	0.946	4	0.237	0.331	0.857
	Error	114.229	89	0.714		

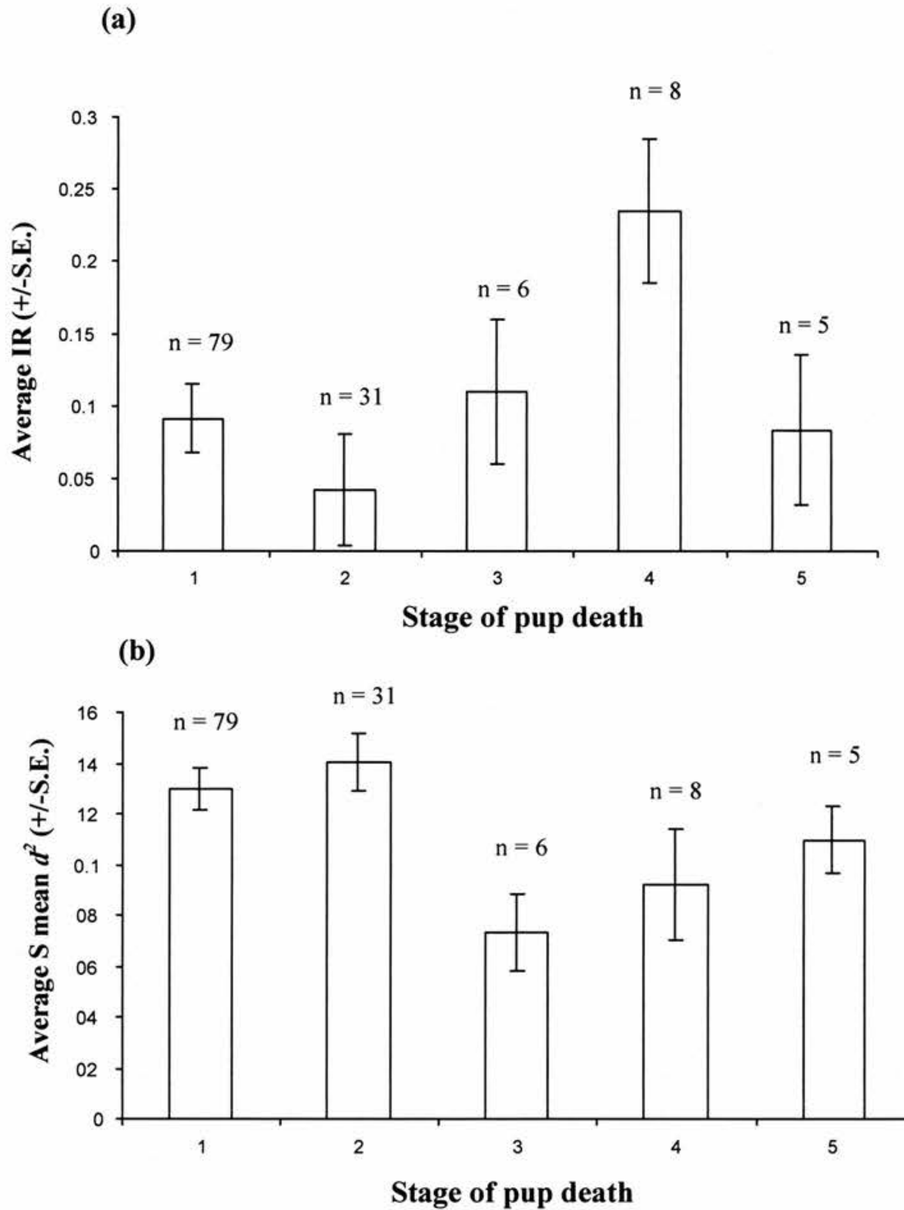

Figure 5.10 **(a)** Average internal relatedness (IR) and **(b)** average standardised mean d^2 (S mean d^2) of the stage at which a pup died on the Isle of May in 2002.

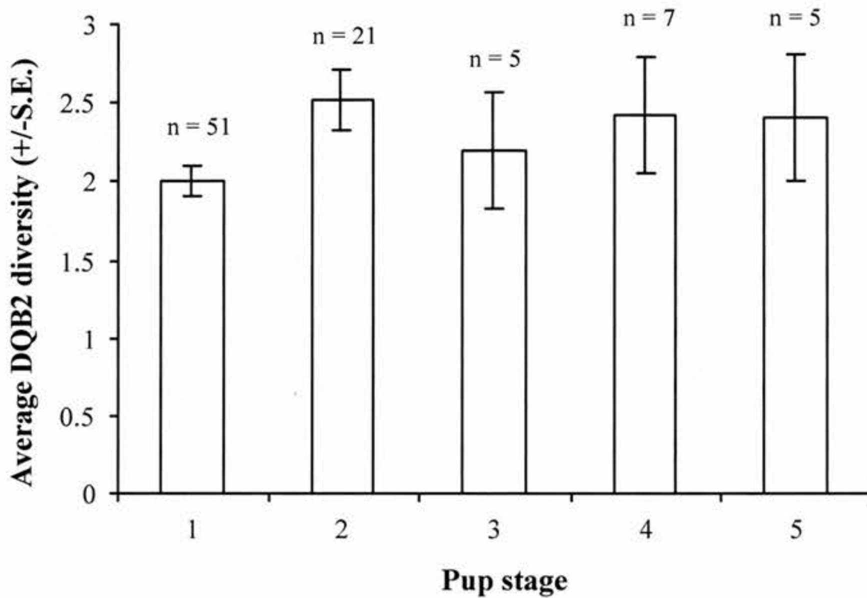

Figure 5.11 Average DQB2 diversity (+/- S.E.) of dead grey seal pups and pup stage on the Isle of May in 2002.

5.3.6 Do all microsatellite loci contribute equally?

So far I have shown that dead pups have lower levels of heterozygosity than live pups. Individual heterozygosity measured using microsatellites is often correlated with fitness, with inbreeding depression often linked as the underlying mechanism. However, more recently it has emerged that heterozygosity/fitness correlations may not reflect inbreeding depression but may instead involve a subset of markers that lie near genes under balancing selection (Balloux et al. 2004). To examine the contribution of the microsatellite loci used here to pup survival I tested each locus separately, fitting a GLM with heterozygotes scored as one and homozygotes as a zero as a categorical factor and pup status (live/dead), as the response variable, modelled with a binomial error structure. Alpha over-inflation was accounted for by sequential Bonferroni correction (Sokal & Rohlf 1995). I found significant effects at four loci, three of which

are significant at $P < 0.01$ (Table 5.10). For all these loci, the effect is in the direction of dead pups having lower heterozygosity. In addition, for all but one locus, the island-locus interaction term was not significant, indicating that these loci tend to show the same trend across all sites (Table 5.10). However, for locus 4.2 the interaction term is significant, suggesting that at this locus the effect may differ between sites (Table 5.10).

Table 5.10 General linear models of the effect of each locus within each island in determining whether a pup is live or dead.

Factor	F	P
Hg3.6	1.284	0.257
Hg3.6 * Island	0.594	0.552
Hg4.2	0.537	0.464
Hg4.2 * Island	4.18	**0.015**
SGPv9	7.441	**0.006**
SGPv9 * Island	1.243	0.288
SGPv11	0.085	0.771
SGPv11 * Island	0.014	0.986
Hg6.1	1.284	0.257
Hg6.1 * Island	0.594	0.552
Hg8.1	4.074	**0.044**
Hg8.1 * Island	0.507	0.602
Hg8.9	1.786	0.181
Hg8.9 * Island	0.352	0.703
Hg6.3	8.092	**0.004**
Hg6.3 * Island	0.319	0.727
Hgd2	7.516	**0.006**
Hgd2 * Island	0.417	0.659

5.4 Discussion

5.4.1 The effect of inbreeding and the DQB2 region on pre-weaning pup survival

Previous studies have shown that high parental similarity reduces juvenile survival in deer (Coulson et al. 1998) and harbour seals (Coltman et al. 1998). My results show that the same holds true in grey seals. When pups are classified simply as live or dead at the time of sampling, IR reveals a consistent pattern across all three islands in which dead pups tend to have higher measures of inbreeding than live pups. Heterozygosity at the MHC region has also been shown to influence fitness in mice (Penn et al. 2002) and higher MHC diversity has been linked to increased fitness in three-spined sticklebacks (Reusch et al. 2001). The levels of heterozygosity and diversity at the DQB2 region in live and dead pups on the Isle of May and North Rona supports these previous studies and reflects results obtained using IR. Not only were there higher levels of DQB2 diversity and heterozygosity in live pups, there were considerable differences between islands. To my knowledge, this is the first evidence that not only is fitness influenced by heterozygote advantage but also by levels of MHC diversity *per se* in a wild population. Increased diversity and heterozygosity in live pups may allow these individuals to deal with infectious diseases more effectively thus supporting the idea that heterozygosity at the MHC is important in determining fitness. The significantly higher frequency of haplotypes 3, and 3,4,5 in dead compared to live pups also suggest that some haplotypes may be more important than others in relation to fitness.

Comparing the size of the effect of inbreeding at the three breeding colonies under investigation suggests that inbreeding effects are somewhat weaker at Sable Island than at the Isle of May or North Rona. Furthermore, this trend is also reflected in the DQB2 analysis, the two UK populations have similar levels of DQB2 diversity and heterozygosity while Sable Island is significantly different. Differences in IR, S mean

d^2 and DQB2 diversity/heterozygosity between the three islands may be due to a number of factors. Firstly, the Sable Island colony is more than an order of magnitude larger than either of the British colonies and individuals do not appear to display as strong year-on-year site fidelity as is the case for the two UK populations (Boness & James 1979). Territory holding males and females on the Isle of May and North Rona have been shown to display high site fidelity (Pomeroy et al. 1994; Twiss et al. 1994; Pomeroy et al. 2000b) which may limit gene flow between populations. Results from this study (see chapter two, Table 2.5 for F_{ST} values) also show considerable genetic differentiation between all three islands supporting the idea that different populations have different levels of inbreeding and levels of DQB2 variation and are likely to show different responses in fitness. Consequently, animals at Sable Island are less likely to mate with relatives due to large population size and low site fidelity resulting in lower levels of inbreeding and higher DQB2 diversity and heterozygosity. Furthermore, even if decreased heterozygosity is associated with pup mortality, the average difference in parental similarity between dead and live pups will be relatively small, making it difficult to detect without much larger sample sizes of both pups and genetic markers.

Secondly, the overall variation between the islands may be strongly linked to colonisation history. Recently founded colonies could potentially have higher levels of DQB2 variation and lower levels of inbreeding if founder individuals arrive from populations that differ in their genetic composition. Furthermore, individuals breeding on new colonies may be more flexible in the mating strategies they use. That is, the potential for mate choice and aquatic mating may be higher, where there is not an established pattern of polygynous mating and where higher rates of consanguineous matings occur. This may well be true for the UK populations. North Rona is a relatively old, established colony and the Isle of May was more recently colonised. As

such, seals from the Isle of May may have higher DQB2 diversity/heterozygosity and lower levels of inbreeding due to the recent colonisation of the island, potentially from genetically dissimilar individuals. This coupled with a higher proportion of individuals participating in mate choice could lead to the higher levels of diversity and heterozygosity observed in this study.

Finally, grey seals in the UK have historically been hunted and culled over a longer period than the Canadian population. The low levels of heterozygosity and DQB2 diversity observed here, compared to the Sable Island population, may also reflect this. Hence, the effect of demographic parameters such as population size, colonisation history as well as mating strategies on DQB2 diversity/heterozygosity and levels of inbreeding warrants further investigation.

5.4.2 The relative importance of inbreeding and the DQB2 region on cause of mortality

In this study, there was no significant relationship between either of the two estimates of inbreeding and any of the causes of mortality. This contrasts with findings by Acevedo-Whitehouse et al. (2003), who showed that in California sea lions, diseased individuals with higher IR values were more likely to succumb to particular causes of death. However, marked differences in levels of inbreeding are discernible between the different categories. For example, pups that had succumbed to infectious agents had lower heterozygosity than pups that had presumably randomly been killed by sudden traumatic events. Detailed diagnostic information may be needed in order to classify cause of mortality into finer categories reducing the variation around these results.

Analysing the effect of the DQB2 region on possible cause of mortality revealed that although DQB2 diversity itself did not vary significantly between different causes

of mortality, the haplotype 2,5 was important in determining an individual's susceptibility to infection. The importance of particular alleles in determining susceptibility to infection is consistent with the general function of class II MHC genes, ie. the binding of extracellular peptides, in particular those produced by bacterial infections (Hughes & Yeager 1998). Furthermore, the percentage of dead pups with haplotype 2,5 was far higher than the percentage of live pups with the same haplotype. It could be suggested that a high proportion of dead pups carrying the 2,5 haplotype on North Rona may have died either directly or indirectly due to infectious agents as has been found to by numerous other studies (see Anderson et al. 1979; Baker 1988; Baker & Baker 1988). A more conclusive study on the effects of possessing a 2,5 haplotype on other islands would require more detailed necropsy data on more pups than were available here.

The lack of a direct effect of DQB2 diversity *per se* in relation to cause of mortality may mean that for the cause of death categories used here, DQB2 diversity does not result in enhanced immune response to particular pathogens. The reason for this may be that most immune responses are dominated by T-cell recognition either of a single or a number of MHC- presented peptides (immunodominance), rather than all or most of the presented peptides (McClelland et al. 2003). Hence, presentation of lots of peptide antigens may have little effect unless the 'correct' ones are presented. For example, in instances where a specific peptide of a particular pathogen is presented but not recognised, the diversity of the rest of the MHC is irrelevant no matter how great it may be. As such carrying rare alleles may be more important than diversity.

My findings also support those presented in a growing body of literature that links particular alleles or haplotypes to susceptibility to particular infectious diseases. For example, in mice, individual MHC alleles from the class I H-2D region can alter the

outcome of infectious diseases such as lymphocytic choriomeningitis virus (Zinkernagel et al. 1985). In humans, particular MHC alleles have been linked to susceptibility to numerous diseases from leprosy and tuberculosis (Hill & Kaufman 1997) to malaria (Hill et al. 1991), and hepatitis virus infections (Thursz et al. 1997), and more recently HIV disease progression (Carrington et al. 1999). To my knowledge, results from my study are the first to provide positive support for the importance of particular alleles/haplotypes in determining susceptibility to infection in a wild population. However, it is unclear what keeps these haplotypes from being eliminated by natural selection. One possibility is that MHC heterozygote superiority emerges over multiple infections because MHC-mediated resistance is generally dominant and many allele-specific susceptibilities to pathogens will be masked by the resistant allele in heterozygotes. This seems to be the most likely mechanism through which deleterious alleles are maintained in the grey seal population.

5.4.3 Internal relatedness versus standardised mean d^2

Over the last five years, several measures of parental similarity calculated from microsatellite data have been explored for their ability to uncover a relationship with measures of fitness in natural populations (Coltman et al. 1998; Coulson et al. 1998; Hoglund et al. 2001; Rowe & Beebee 2003). Theoretical analyses suggest that S mean d^2 is only likely to be informative in a narrow range of circumstances, for example when dissimilar populations meet (Hedrick et al. 2001b; Goudet & Keller 2002; Slate & Pemberton 2002). Such predictions are born out by empirical studies where S mean d^2, despite its initial success, tends to be less informative than measures based more directly on simple heterozygosity (Hedrick et al. 2001b; Goudet & Keller 2002). Where S mean d^2 has been successful in uncovering inbreeding effects, the pattern tends not to

be general, but instead often seems associated with a single locus (Slate & Pemberton 2002). Results from this chapter support these ideas. With respect to inbreeding in grey seal pups, S mean d^2 appears to be less sensitive than IR, and, where there are trends, these are sometimes inconsistent with a simple model in which increased parental similarity leads to reduced fitness. For example, while all causes of death are associated with greater inbreeding for IR, with S mean d^2, three of the five causes yield average values suggestive of outbreeding. Hence, it appears that S mean d^2 is less informative than IR for investigating the relationship between inbreeding and fitness.

5.4.4 Does DQB2 diversity or inbreeding vary with pup age?

Despite my analysis being based on rather small sample sizes, the relationship between inbreeding and pre-weaning pup mortality shows a pattern for IR with a slight peak around weaning. Higher IR values around weaning is unexpected, as it could be expected that IR values should be higher in dead pups just after birth. At this time pups are exposed to pathogens for the first time, mother-pup recognition may be disturbed by other individuals or certain environmental conditions (Fogden 1971; McCulloch et al. 1999) and there may be aggression from surrounding females and males (Smiseth & Lorentsen 2001). However, it may be that weaning may also be a time when stress is high due to changes in physiology and behaviour, and in particular, loss of the immune boost provided by the mother in her milk. Interestingly, dead pups sampled just after weaning had considerably lower levels of inbreeding than pre-weaned pups. This pattern is not reflected using DQB2 diversity or S mean d^2. It is possible that the pattern I found using IR values is caused by low sample sizes, and should therefore be treated with a certain degree of caution.

5.4.5 Are some microsatellite loci more important than others in determining fitness?

The higher levels of IR found in dead pups compared to live pups could be attributable to inbreeding depression associated with an increased number of deleterious recessive alleles. However, when each locus was tested independently, four of the nine loci used were more heterozygous in live pups. Not only did the effect remain after correction for multiple tests, there was no interaction between island and heterozygosity, indicating that the effects were similar across sites. Thus, it seems that several of the microsatellites that were genotyped lie close enough to a gene experiencing a balanced polymorphism for them to show an association between heterozygosity and fitness. Recent work by Balloux et al. (2004), has suggested that if inbreeding is the dominant mechanism, then consanguineous matings need to be vastly more common than is predicted for most populations and that if heterosis explains the pattern observed here there needs to be many more polymorphisms with fitness effects and high levels of linkage disequilibrium than are assumed. Hence, it seems likely that there is a linkage between the loci used here and genes experiencing balancing selection.

5.4.6 Conclusions

Data presented in this chapter indicate that inbreeding in grey seals affects pup survival, varies between populations, is highest in dead pups occurring in the infection cause of mortality category and in pups that die just prior to weaning. In addition, I found that S mean d^2 is not as informative as IR and that certain loci contribute to the effects observed here more than others. DQB2 diversity and levels of heterozygosity were higher in live pups on all three islands and reflect the patterns found using IR. DQB2 diversity did not vary with cause of mortality but, interestingly, haplotype 2,5 seems to

be particularly important in determining an individual's susceptibility to infection. Future studies that include larger sample sizes and examination of other MHC regions, such as class I which is involved in immunity to intracellular pathogens, may help to refine some of the patterns seen here. In addition, examination of the effect of demography on DQB2 heterozygosity and diversity between different islands warrants further investigation.

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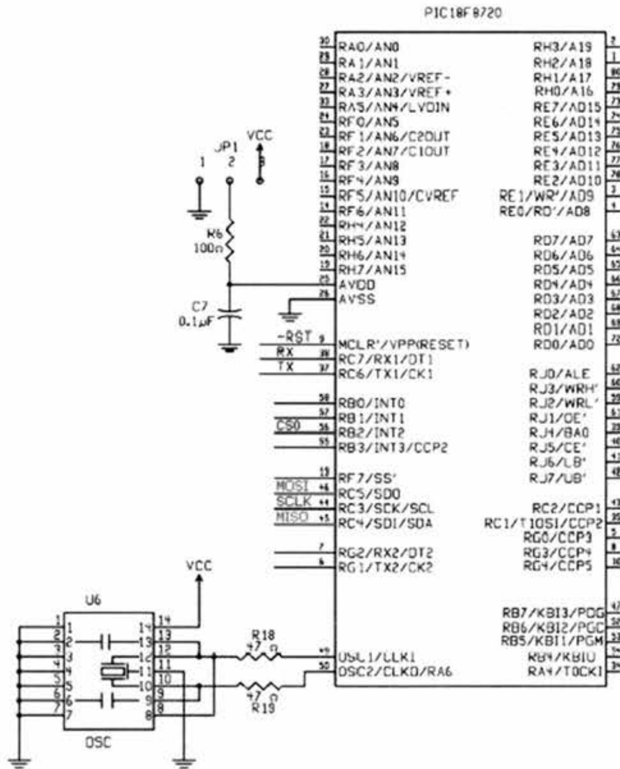
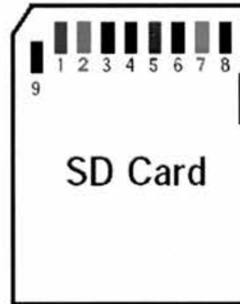
"options.h" file should be included in the main project source file, and the other source files should be located in the directory specified in "options.h" (see the **OPTIONS.H Header File** section for details).

Secure Digital and Multimedia Cards

The Secure Digital card should be hooked up to the SPI bus of the Microchip PIC Processor with the Microchip PIC processor as the master. The test code included with the project assumes a Microchip PIC 18F6720 (for the full demo) or an 18F452/458, (with 3.3V conversions if necessary) is used, with the MOSI, MISO, and SCLK pins of the SD card connected to the SPI bus of the Mega128. CS0 is the card select of the SD card, and could be changed to use any output pin desired (as long as it is properly defined by SD_CS_ON(), SD_CS_OFF(), and CS_DDR_SET() in the "options.h" file).





Pins	SPI Mode		
	Name	IO Type	Description
1	CS	I	Chip Select (Negative True) **(CS0)**
2	DI	I	Data In **(MOSI)**
3	V_{SS}	S	Ground
4	V_{dd}	S	Supply Voltage
5	SCLK	I	Clock **(SCK)**
6	V_{SS2}	S	Ground
7	DO	O/PP	Data Out **(MISO)**
8	RSV	-	Reserved (*)
9	RSV	-	Reserved (*)

Hardware Diagram for Example Project for PIC 18F8720
Interfaced with a Secure Digital Card

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CHAPTER 6

The effect of the DQB2 region on parasite susceptibility in the European wild boar

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The effect of the DQB2 region on parasite susceptibility in the European wild boar

6.1 Introduction

In chapter five, I investigated the relationship between the DQB2 region on offspring survival and cause of mortality in the grey seal. The results suggest that the DQB2 region is important in determining pre-weaning mortality in the grey seal and that particular alleles may confer susceptibility to general infections. This provides the first evidence for a positive relationship between increased DQB2 diversity, heterozygosity and the presence of particular alleles in determining offspring survival in the grey seal. However, I was unable to investigate the role of the DQB2 region in determining susceptibility to specific parasites. Detailed databases which contain information on the infection status of individuals in relation to different parasite types are difficult to collate and require detailed pathological examinations such as serology, histology and microbiology, which were not available for the grey seals examined in this study. Instead, I had access to a dataset for a population of European wild boar in Spain that contained information on the infection status of individuals infected with seven different parasites. Thus, in this chapter I investigate the importance of the DQB2 region in determining susceptibility to parasites in a natural population of European wild boar.

6.1.1 The European wild boar in Spain

The European wild boar (*Sus scrofa*) has undergone considerable range expansion throughout Spain since the 1960s, and now occurs in many areas where it had previously been extirpated or not previously occurred (Gortazar et al. 2000; Fernandez-de-Mera et al. 2003a). Recently, changes in agricultural practices, the introduction of artificial feeding sites and a decrease in the numbers of large predators have resulted in an increase in wild boar numbers (Gortazar et al. 2000; Massei & Genov 2000). Furthermore, the extensive re-introduction of wild boar for hunting purposes is a major factor leading to the increase in overall population size (Fernandez-de-Mera et al. 2003b; Vernesi et al. 2003). Sport hunting has benefited from the re-introductions of wild boar, but increased animal density in relocation areas and at feeding sites has implications for the spread of disease (Gortazar et al. 2003). Not only can disease spread quickly through areas of high wild boar density, it can also spread to extensively bred domestic pigs that share their habitat with the wild boar (Fernandez-de-Mera et al. 2003a).

6.1.2 Disease and parasite susceptibility

In order to maintain healthy populations of wild boar and avert the transfer of diseases to domestic animals, identification of the factors that contribute to pathogen spread and in particular, susceptibility is of particular importance. Although population density is one factor that can contribute to the spread of many diseases, genetic variation is also thought to contribute to disease and parasite susceptibility. As mentioned in chapter five, genes of the MHC are crucial for immune response yet they have also been linked to pathogen susceptibility. For example, a number studies on humans, mice and fish have shown that not only heterozygosity at the MHC, but the presence of particular

alleles decreases susceptibility to infections (Doherty & Zinkernagel 1975; Langefors et al. 2000; Cigel et al. 2003). However, despite the evident importance of genetic factors in relation to pathogen susceptibility in mammals (Potts et al. 1994; Cassinello et al. 2001; Penn et al. 2002; Acevedo-Whitehouse et al. 2003), the economic importance of wild boar and the extensive literature on the diseases and parasites affecting wild boar (Serraino et al. 1999; de-la-Muela et al. 2001; Gortazar et al. 2002; 2003; Rajkovic-Janje et al. 2002; Vicente et al. 2002; Ellis et al. 2003; Fernandez-de-Mera et al. 2003a), only one study has investigated the relationship between disease susceptibility and levels of genetic variation in this species (Acevedo-Whitehouse 2004). Acevedo-Whitehouse (2004) revealed a significant association between bovine tuberculosis (bTB) infection and levels of inbreeding measured using internal relatedness in the same Spanish population of wild boar investigated in this chapter. This study demonstrated that less heterozygous individuals were more likely to be infected by bTB (Acevedo-Whitehouse 2004). Interestingly, two of the microsatellite loci that displayed the strongest effect in relation to levels of inbreeding and bTB infection are located near to the class II MHC gene cluster (Acevedo-Whitehouse 2004).

The European wild boar is an appropriate species in which to investigate the relationship between variation at the MHC and parasite susceptibility. First, wild boar are one of Europe's most important big game species (Vicente et al. 2002) and this provides researchers with access to wild boar tissue samples that may otherwise be difficult to collect from such large and often aggressive animals. Second, the parasites that infect wild boar have been extensively documented and consequently methodologies for detecting different parasite types are well established (Vicente et al. 2002; Fernandez-de-Mera et al. 2003a; 2003b). Third, findings by Acevedo-Whitehouse (2004) which linked genetic heterozygosity, measured using

microsatellites, to infection with bTB suggest that genetic factors, particularly variation at the MHC region may be important in disease and parasite susceptibility in this species. Hence, in this chapter I investigate the relationship between the Class II MHC DQB region (DQB2) and parasite susceptibility in the same Spanish population of hunted wild boar that was previously analysed using microsatellite heterozygosity (see Acevedo-Whitehouse 2004).

6.2 Materials and Methods

6.2.1 Study sites, sample and data collection

The research was conducted from February 2000 to April 2003 in the provinces of Ciudad Real and Toledo in southern Spain (38° 55′ N, 00° 36′ E) (Figure 6.1). The study sites included five fenced hunting estates (closed estates), two open hunting areas (semi-open estate) and one natural park (open estate). The climate is Mediterranean and the habitat is characterised by evergreen oak, scrubland and scattered pastures (Gortazar et al. 2002). Each of the estates differed in management practices, geographic characteristics, wild boar density and the potential for contact with other animals.

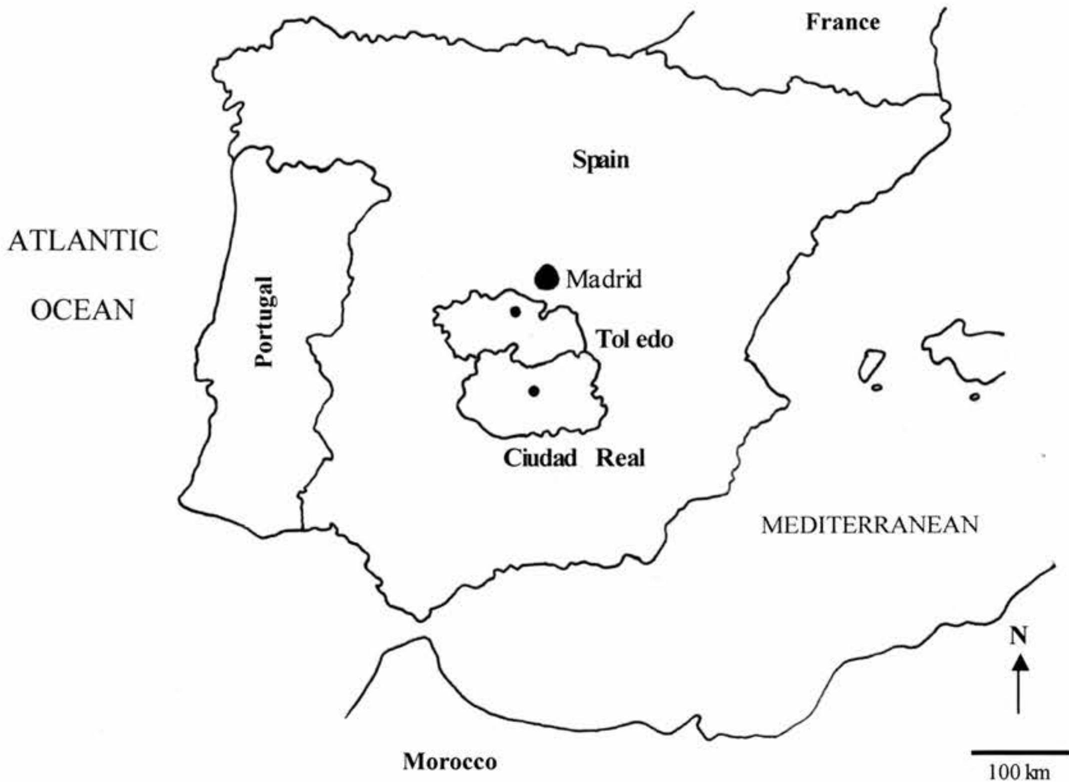


Figure 6.1 Map of Spain indicating the two provinces, Toledo and Ciudad Real from which all wild boar samples were collected.

Data on the presence or absence of seven parasite types in each individual were collected by researchers at IREC (Instituto de Investigación en Recursos Cinegéticos) Spain, as part of an ongoing project on the European wild boar. Tissue samples were taken by IREC staff from 184 wild boars that had been shot by hunters or euthanased in the 2000-2003 hunting season. Sex and age class were recorded and a full necropsy was conducted on all carcasses by IREC staff. I extracted DNA from tissue samples using phenol-chloroform as outlined in chapter two. PCR and genotyping techniques for amplification of the DQB2 region were conducted using procedures outlined in chapter three. Of the tissue samples collected, only 124 could be successfully genotyped for the DQB2 region (alleles 6,9, 15, 16 and 35; see chapter three) and were subsequently used in the following analysis.

6.2.2 Parasite classification

Tongue, lung, digestive tract, liver, gall bladder and renal pelvis were examined by IREC staff for the presence of parasites. Parasites were fixed in 70 % ethanol and cleared with lactophenol blue solution for identification. In addition, coprological analysis by zinc sulfate flotation (specific gravity 1.18) using McMaster chambers (Food 1986) was carried out on faecal material that had been extracted from the rectum of each animal after necropsies had been completed. Parasite identification was based on figures and descriptions given in (Soulsby 1982; Boch & Supperer 1983; Mehlhorn et al. 1992). Using these techniques seven parasite species were identified (Table 6.1). A total of 40 individuals were parasite free, 33 individuals were infected with one type of parasite, 9 individuals were infected with two types of parasite and 3 individuals were infected with three parasite types.

Table 6.1 Total number of individuals infected and not infected with each of the parasites identified.

Parasite	Infected	Not infected
Metastrongylus sp.	38	47
Capillaria garfiai	2	83
Oesophagostomum dentatum	1	84
Ascaris suum	3	82
Coccidia	1	84
Spiriridos sp.	9	76
Macracanthorhynchus hirundinaceus	6	79

6.2.3 Data analysis

Individual DQB2 diversity was expressed as the number of alleles carried by an individual. Haplotypes were coded for by the alleles they contained. To test if sex, age group and estate status co-vary with parasite susceptibility, a GLM was conducted with a normal error structure. Due to inequality of error variances DQB2 diversity data were $\log_{10}(x+1)$ transformed which helped to stabilise variances (Sokal & Rohlf 1995). To determine whether particular alleles were more prevalent in particular estates, the proportion of DQB2 alleles in closed, semi-open and open populations were compared using chi-squared analysis.

Not all individuals were analysed for all parasite classes. Only those which had been tested for all seven parasite types were used ($n = 85$) for analyses of the relationship between the DQB2 region and parasite susceptibility. Analysis of DQB2 diversity and whether or not an individual was infected with parasites was conducted using a Kruskal-Wallis test due to the inability to stabilise variances (Sokal & Rohlf

1995). Variation in the proportion of DQB2 alleles between parasite infected and non-infected individuals was compared graphically.

As well as examining if DQB2 diversity varied in relation to whether an individual was infected or not infected with parasites, I investigate if the number of parasite types an individual is infected with can be explained by DQB2 variation. In order to determine if infection with multiple parasite types varied in relation to DQB2 diversity a one-way ANOVA was conducted. DQB2 diversity was treated as the response variable and individuals not infected and infected with one, two and three parasite types as the fixed factor. The proportion of each of the DQB2 alleles in individuals not infected with parasites and those infected with one, two and three parasites was compared graphically. The proportions of heterozygotes (individuals which carried alleles 35 and 16) and homozygotes in individuals not infected with parasites and individuals infected with one, two and three parasite types were also graphically compared as the low number of heterozygotes ($n = 5$) prevented meaningful statistical analysis.

6.3 Results

6.3.1 Variation in sex, age group and estate type with DQB2 diversity

There is the potential for factors such as age, sex and estate type to vary in relation to DQB2 diversity. To test this, the relationship between DQB2 diversity and sex, age group and estate type was analysed using a GLM. Results from this analysis indicate that there was no significant relationship between sex, age group, estate type and DQB2 diversity (Table 6.3). There was no significant difference in the proportion of each allele in closed, semi-open and open estates (results of chi-squared analysis: $\chi^2 = 4.15$, d.f. =5, $P > 0.05$). However, allele 15 occurred less frequently in closed estates (proportion of allele 15 = 0.9 %) compared with open and semi-open estates (proportion of allele 15 = 7.1 % and 5 % respectively) (Figure 6.2).

Table 6.3 Results of a GLM exploring the possible link between sex, age group, estate type and DQB2 diversity in the European wild boar.

Factor	Type III sums of squares	d.f.	Mean square	F	*P*
Group	0.017	6	0.003	0.353	0.906
Estate type	0.043	2	0.021	2.668	0.076
Sex	0.008	1	0.008	0.976	0.326
Age	0.013	2	0.007	0.804	0.451
Error	0.642	80	0.008		

Investigation of haplotype frequencies between each of the three estates revealed that haplotype 6,35 occurred considerably less often in open estates compared to semi-open and closed estates (Figure 6.3). Haplotype 35 was more prevalent in closed estates

compared with open and semi-open estates, and there were more individuals with haplotype 35,9 in open estates compared to semi-open or closed estates (Figure 6.3).

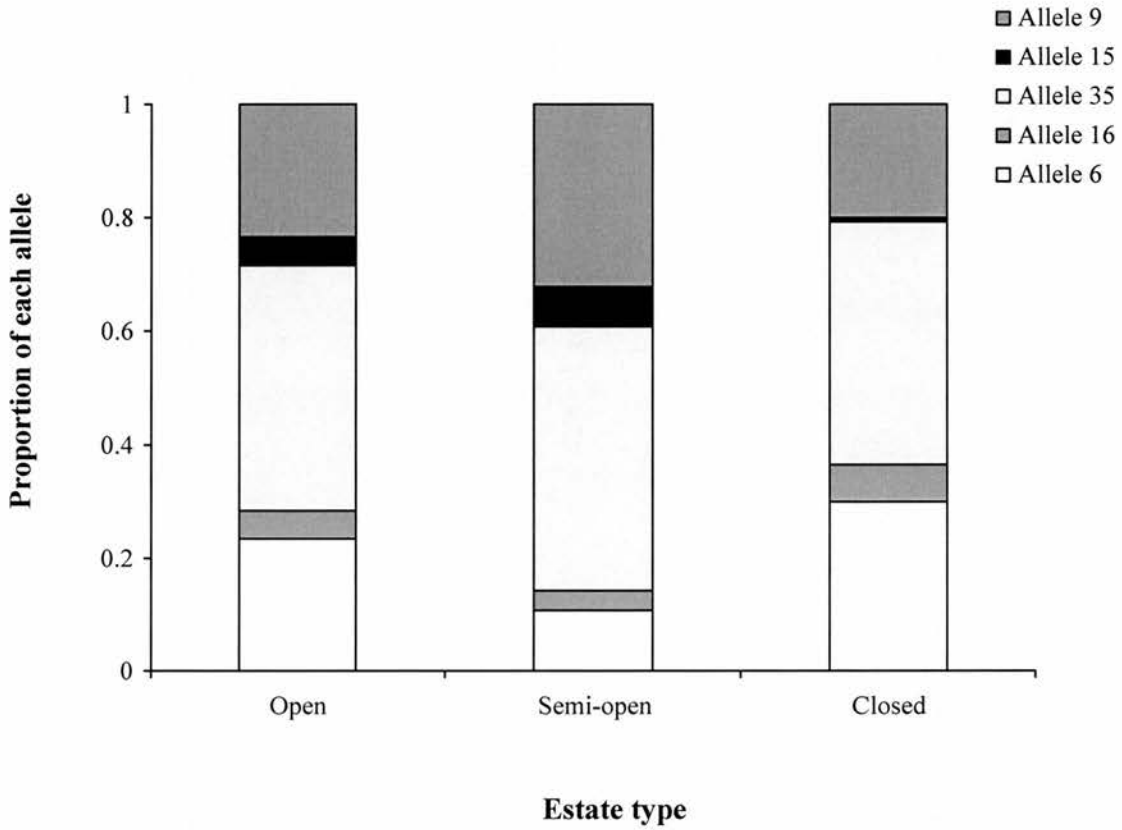


Figure 6.2 The proportion of DQB2 alleles 9, 15, 35, 16 and 6 in relation to estate type: open, semi-open and closed, in wild boar.

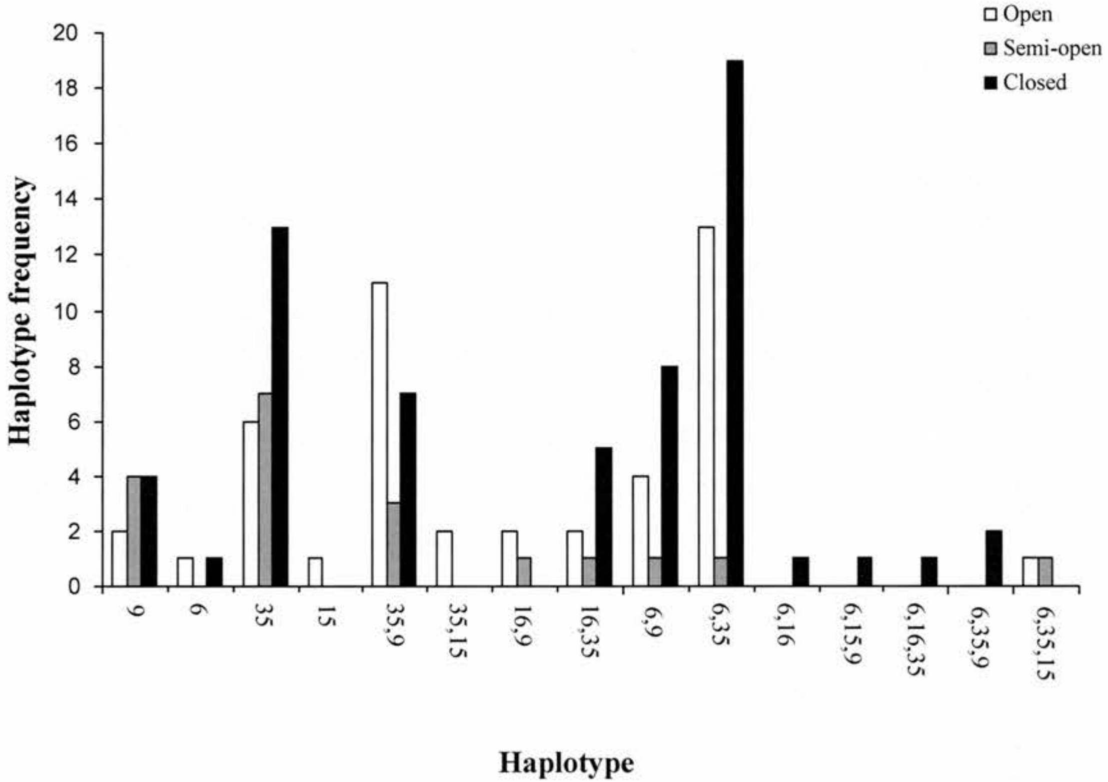


Figure 6.3 DQB2 haplotype frequencies of wild boar from open, semi-open and closed estates.

6.3.2 The effect of the DQB2 region on parasite susceptibility

There was no significant effect of DQB2 diversity on whether or not an individual was infected with parasites (results of a Kruskal-Wallis test: $\chi^2 = 1.686$, d.f. = 1, $P = 0.194$) (Figure 6.4). When the proportion of DQB2 alleles in individuals infected and not infected with parasites were compared, parasite infected individuals had a considerably lower proportion of alleles 16, 35 and 15 and a higher proportion of allele 6 (Figure 6.4).

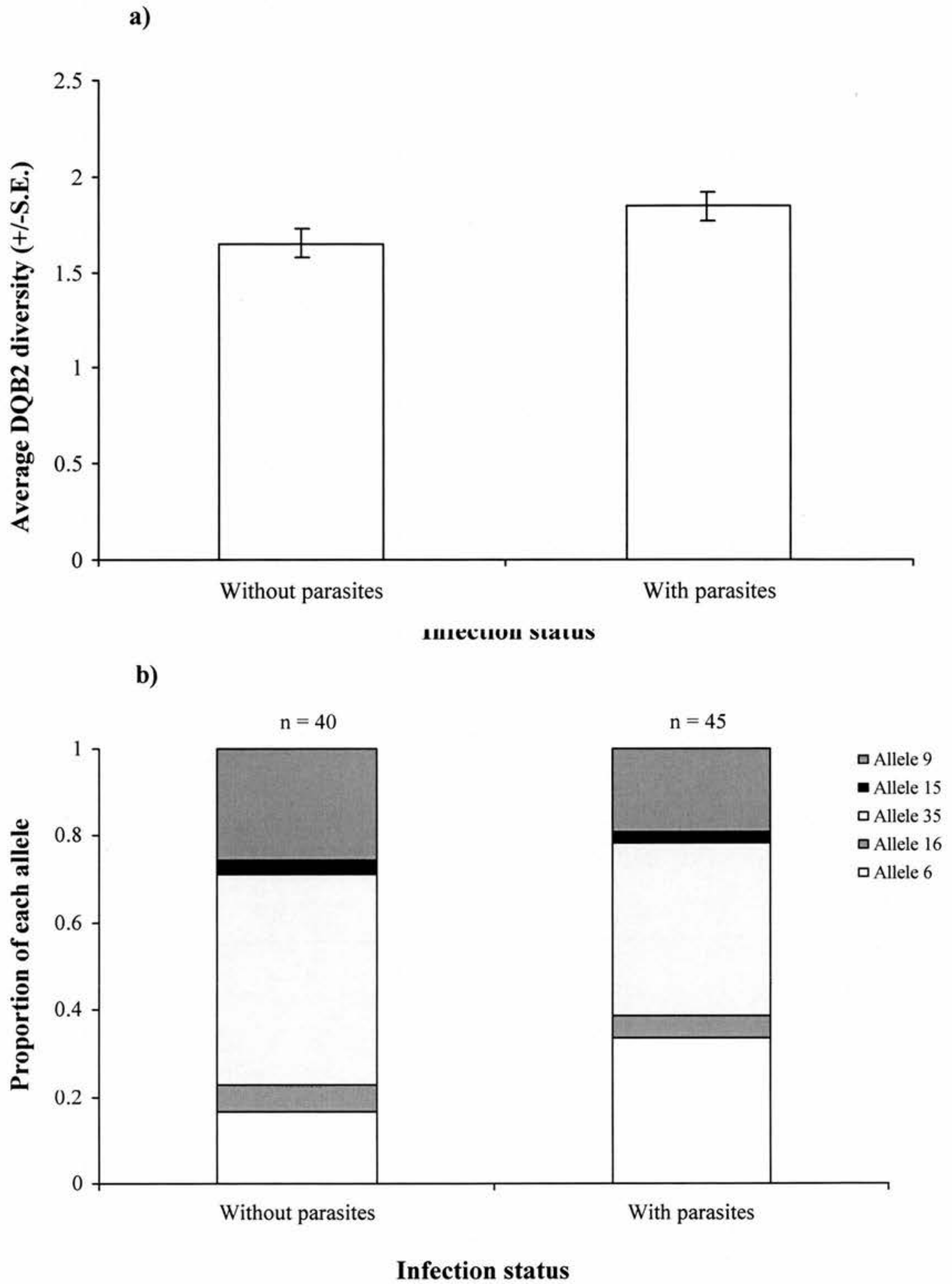
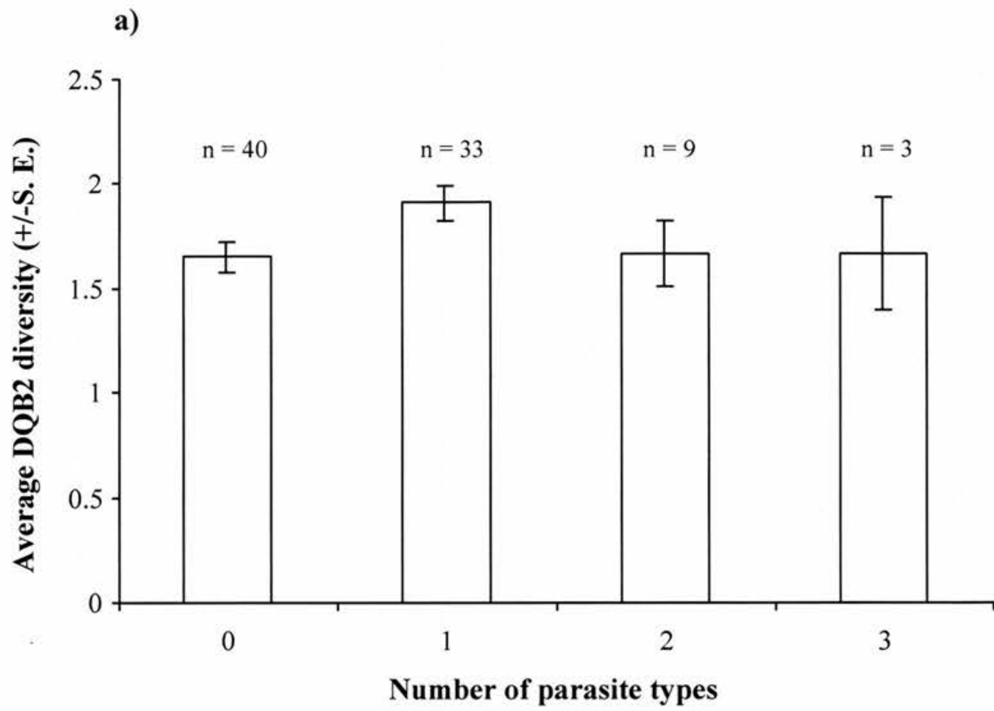


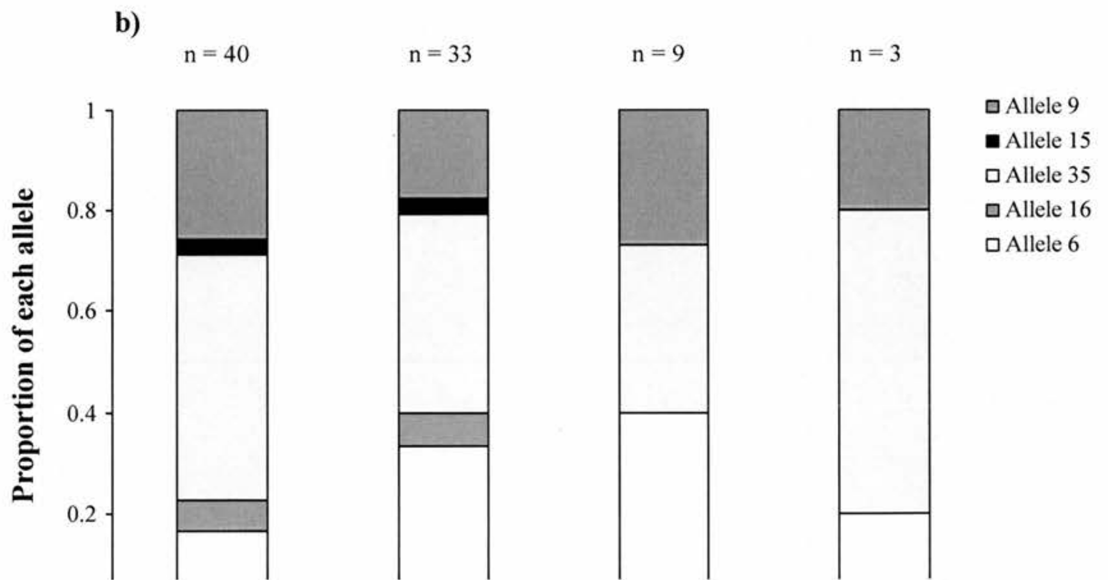
Figure 6.4 **(a)** Average DQB2 diversity (+/- S.E.) and **(b)** the proportion of DQB2 alleles 9, 15, 35, 16 and 6 in wild boar infected and not infected with parasites. Sample sizes are indicated above each bar.

There was no significant difference in DQB2 diversity between individuals not infected with parasites and those infected with one, two and three parasite types (results of one-way ANOVA: $F_{3,81} = 1.74$, $P > 0.05$) (Table 6.4). However, in individuals that were not infected with parasites or those that were infected with only one type of parasite all five DQB2 alleles were present (Figure 6.5). In contrast, in individuals infected with two or three parasite types only three DQB2 alleles (alleles 6, 35 and 9) were present (Figure 6.5). Furthermore, there were considerably more heterozygous individuals amongst those not infected with parasites or infected with only one type of parasite compared to individuals infected with two or three parasite types (Figure 6.6). The proportion of homozygotes in individuals infected with two and three types of parasite was 100 % compared with individuals not infected with parasites (92.5 % homozygotes) or those infected with only one type of parasite (93.94 % homozygotes) (Figure 6.6). Individuals infected with parasites had a considerably higher frequency of haplotypes 6,35 and 6,9, while those not infected with parasites had a higher frequency of haplotypes 35 and 35,9 (Figure 6.7).

Table 6.4 Results of a one-way ANOVA between DQB2 diversity and the number of parasites types carried by an individual.

Factor	**Type III sums of squares**	**d.f.**	**Mean square**	**F**	***P***
DQB2 diversity	1.318	3	0.439	1.736	0.166
Error	20.494	81	0.253		





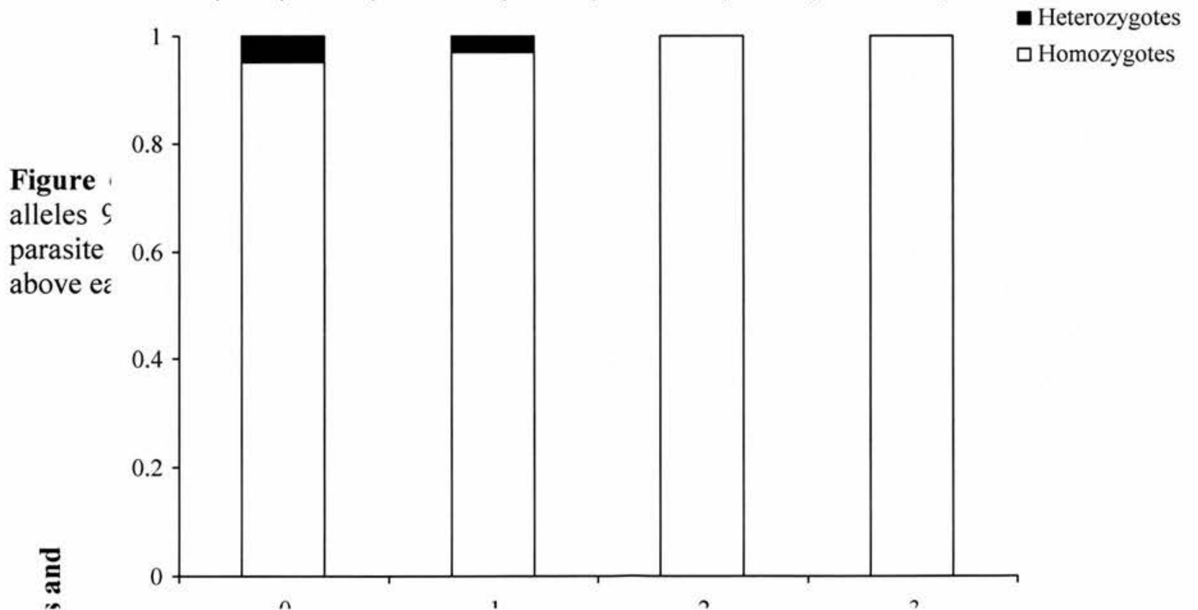


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Figure 6.6 The proportion of homozygotes and heterozygotes in wild boars not infected with parasites and those infected with one, two and three parasite types. Sample sizes are indicated above each bar.

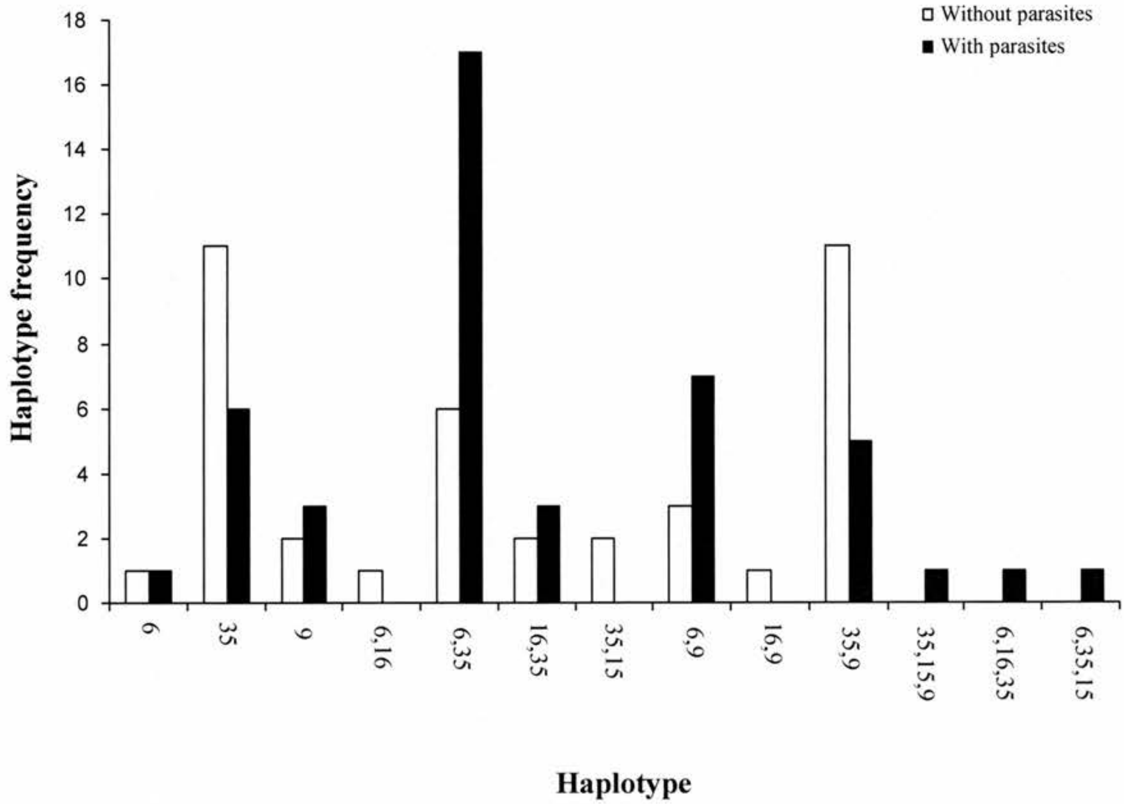

Figure 6.7 Haplotype frequencies of wild boar infected with parasites and individuals free from infection from all estates combined.

6.4 Discussion

In this chapter I investigate the relationship between the DQB2 region and parasite susceptibility in a Spanish population of wild boar. There were no notable differences in DQB2 diversity or the proportion of particular alleles between the three estate types. Overall levels of heterozygosity and diversity were very low in the population studied here. There are currently 16 published alleles from the DQB region in the genus *Sus* (GENEBANK: http://www.ncbi.ac.uk). However, using DGGE I was only able to resolve 5 DQB2 alleles. Such low diversity and heterozygosity may be due to a number of factors. Firstly, wild boar in Spain have been extensively re-stocked over the last 20 years (Gortazar et al. 2000). If founding individuals of new wild boar populations were taken from a single small population or from a captive population, levels of diversity and heterozygosity in re-stocked populations may be lower than would otherwise be expected in a large wild population, leading to the founder fitness effect (Hartl & Clark 1997). Secondly, DGGE may not be detecting all alleles present. Although numerous checks were made, the technique is not 100 % accurate in detecting all single base changes and as such there exists the small possibility that an allele could have been missed (Cariello & Skopek 1993). However, even if this were to be the case, DQB2 diversity and heterozygosity in this population would still be remarkably low compared to other terrestrial mammals.

All individuals collected for this study were or had been infected with other diseases ie. bovine tuberculosis, bucellosis, porcine circovirus, Aujeszky's disease and porcine parvovirus (Vicente pers.com). Although it is almost impossible to sample a wild individual that is free from infection, comparison of infected and non-infected individuals is problematic without an adequate control. There is no reason for diseased individuals to be preferentially killed by hunters, although it is possible that individuals

that are infected with a disease may not be able to flee from hunters as quickly and may be less alert to danger than healthy individuals. Assessing overall levels of animal health may aid in separating out individuals that are relatively free from infection. These individuals could then be treated as a control group. More importantly, results from this study suggest that management decisions which involve the re-location and introduction of new stock to new or existing areas may need to consider genetic factors in order to minimise parasite load in wild boar as well as the spread of parasites to other animals and indeed humans.

Previous studies on mice (Penn et al. 2002), fish (Langefors et al. 2000; Lohm et al. 2002) and humans (Horn et al. 1988; Carrington et al. 1999) have revealed that the MHC can influence an individual's susceptibility to particular pathogens. Results from this study suggest that a similar mechanism may operate with respect to parasite infection in wild boar, but DQB2 diversity *per se* appears less important than heterozygosity and the presence of particular DQB2 alleles in determining susceptibility to infection with multiple parasites. There was no significant difference in DQB2 diversity between individuals infected or not infected with parasites. However, when individuals were classified according to the number of parasite types that they were infected with, there were considerable differences in DQB2 allele composition between the different categories. One possible explanation for the pattern of allelic composition observed is that DQB2 alleles 9, 35 and 6 increases an individual's susceptibility to multiple infections. These results are similar to those described in chapter five, where haplotype 2,5 was found to increase the susceptibility to general infections in grey seals.

In addition to there being considerable variation in the proportion of particular alleles in individuals not infected and those infected with one, two and three parasite types, there were considerable differences in the proportion of heterozygotes and

homozygotes between these groups. Wild boar infected with two and three types of parasite were composed entirely of homozygotes while those not infected with any parasites had the highest levels of heterozygosity. Unfortunately, low sample numbers prevent a definitive conclusion. However, this finding supports the 'heterozygote advantage' hypothesis, which states that heterozygous individuals are at an advantage due to increased ability to recognise a wider range of pathogens (Hughes et al. 1994). (Penn et al. 2002) reported similar results to those presented here when investigating MHC heterozygosity in relation to multiple-strain infections in mice. This study revealed that of the individuals infected with multiple-strains of *Salmonella spp*, heterozygotes had greater survival than homozygotes and higher rates of *Salmonella spp.* clearance (Penn et al. 2002). Similarly, a recent study by Harf & Sommer (2005) found that the number of DRB exon 2 alleles in the hairy-footed gerbil (*Gerbillurus paeba*) had no effect on whether an individual was 'infected' or 'not infected' with endoparasites. Furthermore, one allele (Gepa-DRB*15) only occurred in uninfected mice thus supporting results in this study where alleles 15 & 16 were only found in uninfected wild boar and individuals infected with one parasite type. Greater sample sizes for individuals infected with two and three types of parasites will allow the tentative results obtained in this study to be statistically investigated.

In this chapter I investigated the relationship between the DQB2 region and parasite susceptibility in the wild boar. The low levels of DQB2 heterozygosity documented here are surprising and future work investigating heterozygosity at other regions is important for the future management of this species. Results from this chapter indicate that there is no significant association between DQB2 diversity *per se* and parasite susceptibility in the wild boar. However, particular DQB2 alleles and levels of heterozygosity may be important in determining parasite species richness. In

particular, work on MHC class I genes in relation to parasite susceptibility and increased samples sizes may also prove fruitful and refine some of the patterns described here.

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CHAPTER 7

General conclusions and future work

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General conclusions and future work

This thesis describes a study of the importance of the DQB2 region on mating strategy in the grey seal. In addition, I investigated the extent to which levels of inbreeding, measured using a panel of nine microsatellites, and variation at the DQB2 region, measured as DQB2 diversity, heterozygosity, the presence of particular alleles and haplotype frequency affect pre-weaning pup survival in the grey seal. Furthermore, I also examined the role of the DQB2 region in relation to disease and parasite susceptibility in the European wild boar. In this chapter, I discuss the wider implications of my findings and make recommendations for future work.

7.1 Genetic variation between three grey seal breeding colonies

Perhaps the single most important recommendation for future research to result from my studies is to relate MHC variability to the effect of colony structure both in terms of colony demographics and the spatial pattern of colonisation. This is because one of the most important findings of this thesis is the significant difference in both levels of inbreeding and DQB2 variation between the three grey seal breeding colonies, the Isle of May, North Rona and Sable Island. As previously documented by Allen et al. (1995) and again in chapter two in this thesis, all three island populations are genetically distinct with apparently no exchange of individuals between the colonies. Since the MHC genes are involved in immune response, and because there are specific interactions between particular MHC alleles and certain pathogens, pathogen exposure is likely to contribute significantly to differentiation between colonies such as those I observed in this study. Furthermore, behavioural and demographic parameters may also

affect our ability to detect selection at the MHC. Since MHC loci evolve within populations they are subject to the same evolutionary forces ie. genetic drift, that affect other loci such as microsatellites (Hartl & Clark 1997). As effective population size decreases, the ability of natural selection to overcome genetic drift is decreased and the effect of these forces may vary between populations with the result that conspecifics in different populations may experience different levels of selection on MHC loci. As such, the history of colony expansion indicated by demographic variation and the way in which individuals mix and choose mates, which is itself affected by the topography of individual islands, needs to be considered in future studies.

7.2 Mating strategies in the grey seal

The fourth chapter in this thesis documents DQB2 variation on the Isle of May and North Rona in relation to mating strategy. The most interesting result to emerge from this research is that on the Isle of May a female's age and/or past mating experience may influence the mating strategy she uses. It appears that initially females may participate in polygyny due to behavioural or physical constraints, and that as a female gets older she may be more likely and more able to choose DQB2-dissimilar mates. This pattern is not found in females breeding on North Rona although there are significant differences in levels of DQB2-dissimilarity between pupping events. Worthington Wilmer et al. (1999) suggested that due to the topography of the Isle of May males are not able to maintain central positions within the colony to the same extent as on North Rona. North Rona females may be more likely to participate in a polygynous mating system rather than exercise mate choice as they appear to do on the Isle of May. Hence, topography may be one factor that could contribute to the pattern observed.

As well as female age and past mating experience, demography may also be important in determining the relationship between MHC variation and mating strategy. For example, in a population founded by only a few MHC-similar individuals, or one that has undergone a dramatic reduction in size, MHC variation between individuals may be particularly low. In such cases, choice for dissimilar partners may be difficult and as such the default reproductive strategy may then be polygyny. However, as time passes and new individuals mix with the existing colony and/or new alleles arise through point mutations there is the potential for levels of MHC dissimilarity to increase such that choice for MHC-dissimilar individuals may occur. The effect of demography on levels of MHC variation and the ways in which this affects mating strategy could be investigated in grey seals breeding on the Orkney Isles in the North of Scotland. The Orkneys consist of a number of small islands that vary in grey seal population size and colonisation history. Grey seals have only recently started to breed on some of the islands. For example, Stronsay, Copinsay and Calf of Eday were either founded or recolonised in 1992. Information on colonisation history of newly founded populations compared with those that are well established, in conjunction with population size, breeding behaviour and levels of MHC-similarity could potentially be used in a model to investigate the relationship between these variables.

One problem with the comparisons between DQB2 variation and mating strategy in the grey seal made in this thesis is that it was not possible separately to analyse DQB2 variation in pups that were conceived as a result of female mate choice and those that were conceived as a result of polygynous matings. Detailed long-term behavioural observations of focal females and males would aid greatly in estimating which mating strategy a female uses each breeding season. This information could then be used in conjunction with the MHC-genotypes obtained from males, females and their

pups, potentially allowing each mating strategy to be investigated separately. This would reduce some of the variation and ambiguities in the results obtained in this thesis.

7.3 Pre-weaning pup survival in the grey seal

In chapter five I used two different genetic estimates of inbreeding in conjunction with variation at the DQB2 region to investigate a possible relationship between these measures with pre-weaning mortality in grey seal pups. The results link decreased heterozygosity with lower rates of survival in pre-weaned pups, and some loci are found to contribute to this effect significantly more than others. This finding suggests that some of the microsatellites used here lie close to a gene, or genes, experiencing a balanced polymorphism. For these loci, it would be interesting to investigate where in the genome these loci are and what genes they lie next to. Unfortunately this is not yet possible as the grey seal genome has yet to be mapped. Perhaps future studies will allow for such investigations to occur.

DQB2 diversity and levels of homozygosity also affect pup survival and vary significantly between the three populations. This suggests non-random mating with respect to MHC haplotypes. However, as indicated above, it is difficult directly to ascertain which individuals participate in mate choice and so the extent to which this takes place is uncertain. In addition, when cause of mortality is broken down into a range of different causes, infection appears to be the cause of death most strongly influenced by reduced heterozygosity. In contrast, individuals carrying only DQB2 alleles 2 and 5 are more susceptible to infection and this haplotype is significantly more common in dead pups than in live individuals on North Rona and Sable Island. However, because this study only encompasses one breeding season there are limitations on the extent to which this finding can be applied to grey seals in general.

Future work which includes pathological examination and sampling of dead pups over a number of breeding seasons would allow for investigation of any year-on-year variation in cause of mortality and the prevalence of particular DQB2 alleles, diversity and heterozygosity.

7.4 Parasite susceptibility in the European wild boar

In chapter six I investigate if variation at the DQB2 region influences susceptibility to disease and pathogens in the European wild boar. Results from this chapter suggest that neither DQB2 diversity, heterozygosity nor the frequency of particular haplotypes affect an individual's susceptibility to infection with parasites. Interestingly, analysis of DQB2 heterozygosity and the presence or absence of particular alleles shows that individuals infected with multiple parasites are invariably completely homozygous and only ever carry a maximum of three of the potential five DQB2 alleles. Unfortunately, low sample size for individuals infected with two and three different parasite types prevents a clear conclusion. However, if these results are indeed correct then they provide support for the theory that increased pathogen resistance is due to individuals being heterozygous at their MHC and that particular alleles may also be important. Increased sample sizes may help to determine if the patterns observed here support the heterozygote advantage hypothesis and whether particular alleles confer protection and/or susceptibility to multiple infections.

Low levels of heterozygosity in the wild boar sampled in this thesis suggest that future management decisions regarding the re-introduction of this species need to consider genetic factors in order to reduce pathogen loads and consequent expensive and wasteful loss of huntable stock. If homozygotes are more susceptible to multiple infections, then re-introductions that include individuals from different populations may

increase genetic heterozygosity and may aid in reducing parasite load in wild boar and in turn transmission to other wildlife. Conservation of large mammalian wildlife not only in Spain but throughout Europe is of particular importance as many species are now in decline (Villafuerte et al. 1994; Nowell & Jakson 1996; Lucchini et al. 2004). For example, the Iberian lynx (*Lynx pardinus*) which is now one of the most endangered felids in the world may be particularly at risk of being infected with diseases affecting wild boar (Nowell & Jakson 1996). This is due to the reduction in their main prey species, the European rabbit (*Oryctolagus cuniculus*) which has resulted in an increased consumption of alternative prey such as wild boar (Villafuerte et al. 1994). This change in prey species has the potential to increase the transmission of diseases from wild boar to Iberian lynx.

7.5 Future work

7.5.1 Technical improvements

One of the limiting steps in genotyping individuals for MHC is the optimisation of DGGE. Although this technique does identify the majority (approximately 98 %) of single base changes in a fragment of DNA (Fischer & Lerman 1983) it is very time consuming in both the optimisation for a given species as well as during genotyping. For example, only 16 samples can be run on a single parallel DGGE at any given time and only two gels fit onto the BIORAD apparatus for electrophoresis. Optimal allele separation of DQB2 alleles occurred after five hours. Gel preparation takes one hour and gel staining another half an hour. Hence, it takes approximately seven hours to genotype only 36 samples. In order to reduce the amount of time it takes for electrophoresis, a reduction of the denaturing gradient may aid in separating alleles over a shorter period of time. Even if this is successful, there are other problems with DGGE

that make the use of alternative techniques more appealing. For example, part way through the genotyping of grey seal samples on parallel DGGE I encountered a number of problems with primer quality affecting the clarity of bands. DGGE is extremely sensitive and I found that even slightly reduced primer quality greatly affected my ability to discern different DQB2 alleles. Hence, due to the time it takes to genotype a large number of individuals using DGGE and the variation in the quality of the bands obtained I recommend two alternative techniques.

First, direct sequencing of MHC alleles has become a realistic option for studies using large sample sizes. Until recently this technique was extremely expensive. However, the cost of sequencing large numbers of samples is now greatly reduced, it is quicker than DGGE to optimise and up to 96 samples can be loaded onto a single sequencing gel. The second technique involves using allele-specific primers (Myakishev et al. 2001; Aitken et al. 2004). This technique involves designing a series of allele-specific primers that can then be used in a PCR to amplify each allele. PCR product could then be electrophoresed through a standard agarose gel where each MHC allele should appear as a band much like on a parallel DGGE. I believe that either sequencing or use of allele specific primers would be more effective techniques for genotyping MHC alleles than DGGE and should be implemented in future research projects.

7.5.2 Cause of mortality assignment and MHC analysis

A problem with comparisons between the cause of mortality and genetic variation is that the actual cause of death can be difficult to determine. Furthermore, cause of mortality can only be accurately determined after detailed pathological examination has been made in conjunction with detailed gross necropsies (Gulland 1997). Assignment

to a cause of mortality category may also vary from person to person. Unless the researcher is an experienced veterinarian or has long term experience in conducting necropsies, the ability to recognise abnormal features can be limited to those that are extremely obvious. Due to these limitations, the cause of mortality categories used in chapter five were very broad. Ideally, future studies investigating cause of mortality in grey seals should aim to include not only detailed necropsy information but also tissue and blood samples taken for pathological examination. Alternatively, because the majority of dead grey seal pups succumb to only a few causes of death detailed guidelines on conducting 'in field' diagnosis of cause of mortality may aid future researchers in determining cause of mortality more accurately as well as standardising results between individuals and studies.

Not only is detailed diagnostic information highly desirable when investigating the cause of mortality in relation to genetic variation, the subsequent analysis of the specific pathogens involved is also extremely important. Although there is overwhelming evidence that supports comparisons between genetic variation and specific pathogens (see Klein & O'Huigin 1994; Read et al. 1995; Acevedo-Whitehouse et al. 2003; McClelland et al. 2003) such analysis may limit us in our understanding of how genetic variation can effect pathogen susceptibility. For example, in chapter six I compare levels of DQB2 variation in wild boar that are infected or not infected with specific diseases. However, is it useful only to make comparisons between a measure of genetic variation and a single pathogen? For example, for any given individual they could be infected with X, Y and Z diseases or pathogens. Comparing only one of these against a genetic measure may not provide an indication of animal health and immune system stress. Therefore, I propose that in addition to comparisons between genetic variation and whether an individual is infected or not infected with a specific pathogen,

analyses should also involve a measure of 'animal health' taking into account as much other information as possible. This could include measures of body condition combined with an indicator of disease/parasite load within a multivariate statistical framework. This approach could allow investigation of the contribution made to variation in health caused by a single pathogen in the presence of different genetic covariates.

7.5.3 Kin recognition

In this thesis I investigate two of the main factors thought to be affected by the DQB2 region, mate choice and pathogen susceptibility. Another factor believed to be influenced by MHC variation is kin recognition (Manning et al. 1992). To date, previous studies have failed conclusively to identify kin association in grey seals despite studies documenting a high incidence of daughters pupping close to their mothers (Pomeroy et al. 1994; 2000b). Since an individual's MHC genotype is believed to be able to be detected by conspecifics through odour cues in urine, this provides a mechanism for individual kin recognition as well as mate choice. Over the past few years urine samples have been collected from focal females on both the Isle of May and North Rona and the females' positions within the colony recorded. Use of behavioural observations and MHC genotypes of females and their daughters may aid in detecting if MHC-correlated kin association in grey seals occurs. Examination of different urinary proteins may also provide an indication of whether females can detect different MHC haplotypes olfactorily.

7.5.4 Post-weaning grey seal mortality

In chapter five I investigated pre-weaning grey seal mortality in relation to levels of inbreeding and variation at the DQB2 region. Although both these genetic measures provide information on survival to weaning, the ways in which inbreeding and DQB2 variation may influence post-weaning pup survival are still unclear. A previous study by Hall et al. (2002), revealed that increased body condition or mass at weaning can be an important determinant of first year survival in the grey seal. Further studies by King et al. (1994); and Hall et al. (2002) have suggested that immunoglobulin levels may be equally important. For example, higher post-weaning circulating serum gammaglobulin (IgG) concentrations have been correlated with reduced first year survival (Hall et al. 2002). Although these factors appear to contribute to first year survival in grey seals, no study has yet examined the relative contribution that levels of inbreeding and the MHC region may have in determining first year survival. Such research would be an interesting extension of work presented in chapter five.

The genetic factors that may contribute to post-weaning survival are currently being investigated as part of a collaboration between Dr Ailsa Hall, Dr Bernie McConnell (Sea Mammal Research Unit, St Andrews University) and myself. During the 2002 breeding season on the Isle of May weaned pups were skin sampled and blood samples taken for analysis of immunoglobulin levels. Using techniques outlined in chapter two and three, each individual was genotyped for the same nine microsatellites and the DQB2 region. A radio tag was fitted to the top of the pup's head and three coloured dots (a mixture of paint and an epoxy resin) were placed on the side of the head to enable tag loss rates to be determined. Each tag is designed to relay a signal every time a pup surfaces for approximately one year. This information will be used to calculate a probability of survival at the end of the one year period. For example, it is

assumed that the pup is dead if a tag continuously relays a signal or ceases to transmit before the end of its one-year lifespan. In order to account for tag loss, surveys of haulout sites were conducted to identify those individuals that had three coloured dots but no tag. All this information will then be included as part of a joint live-recapture/live-resighting/dead-recovery mark-recapture model. This will potentially allow us to investigate the effect of inbreeding, the DQB2 region, immunoglobulin levels and body condition on the probability of first year survival.

7.6 Conclusions

Work presented in this thesis provides the first information on the role of the MHC region and levels of inbreeding in relation to mating strategy and pup survival in the grey seal and the importance of the MHC region in parasite susceptibility in the European wild boar. Examination of other grey seal colonies that vary in their demography, the inclusion of behavioural observations and refinement of techniques used to genotype samples for MHC regions and assignment of cause of mortality will further increase our understanding of how genetic variation affects mate choice and offspring survival. Furthermore, investigation of other MHC regions and increased sample sizes may also aid in determining the relative importance of the MHC on pathogen susceptibility in natural populations.

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