Insect bite hypersensitivity in horses: genetic and epidemiological analysis

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Insect bite hypersensitivity in horses: genetic and epidemiological analysis

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Thesis

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Abstract

Insect bite hypersensitivity (IBH) is the most common allergic skin disease in horses and is caused by bites of *Culicoides* spp. IBH reduces welfare of affected horses and at present no effective preventive measure or cure exists. Aim of our research was to increase knowledge of the genetic background of IBH in horse populations and to explore opportunities to reduce IBH prevalence through selection and breeding.

Data on Shetland pony and Friesian horse mares were gathered at obligatory inspections. IBH prevalence was 7.5% in Shetland pony mares and 18.2% in Friesian horse mares. Data were analyzed to identify risk factors. Combined effect of month and year of IBH scoring, region within the Netherlands and inspector were associated with IBH in both breeds. IBH prevalence significantly differed with coat colour and withers height category in Shetland pony mares. Moreover, prevalence was higher in Shetland pony mares with high body condition score (9.4%).

Quantitative genetic analyses revealed substantial genetic variation for IBH in both breeds. Heritability on the observed scale and on the underlying scale was 0.08 and 0.24 respectively in Shetland pony mares, 0.07 and 0.16 respectively in Friesian horse mares. Therefore, IBH is a heritable phenotype in both breeds. Repeatability was 0.30 in Shetland pony mares and 0.89 in Friesian horse mares. Maternal effect (0.17) was estimated in Friesian horse mares only.

To identify genomic regions contributing to the genetic variance, Shetland pony mares and Icelandic horses were selected according to a matched case-control design. Odds ratios of allele substitution effects of the unfavourable allele were between 1.94 and 5.95. Also, 13 and 28% of genetic variance was explained by all SNPs in respectively Shetland pony mares and Icelandic horses. Significant associated genomic regions across breeds suggest interesting candidate regions on ECA3, 7, 11, 20 and 23 contributing to genetic variance. Results support that ELA class II region on ECA20 is involved in IBH etiology, although follow-up studies are needed to confirm this and to identify genes in the other regions.

The general discussion explored possibilities to reduce IBH prevalence through breeding and discussed implications of using clinical symptoms or diagnostic test results. Simulated selection was based on EBV, which included own performance, progeny performance or genomic data. Selection on IBH clinical symptoms should be based on testing at least 10 but preferably more progeny, accompanying strict selection in sires to achieve reasonable genetic gain. Expected genetic gain per year in genomic selection outperformed other strategies, although implementation of genomic selection requires a considerable investment in a reference population. A diagnostic test for IBH (yet unfeasible to perform on a large sample) has the potential to increase genetic gain.

Contents

1

General introduction

1.1 Introduction

1.1.1 Insect bite hypersensitivity

Insect bite hypersensitivity (IBH) is an allergic reaction of horses to bites of *Culicoides* spp. and is observed in many countries worldwide. IBH is also known as "summer eczema", "sweet itch", "Queensland itch", "allergic dermatitis" or "kasen" (Riek, 1954; Nakamura *et al.*, 1956; Braverman *et al.*, 1983; Broström *et al.*, 1987; Eriksson *et al.*, 2008). IBH is characterized initially by papules followed by severe itch, which is considered the most common clinical symptom (van den Boom *et al.*, 2008). The itch results in self-inflicted trauma causing hair loss, scaling, crusting, thickening of the skin and possible open wounds occurring primarily at the base of the tail and crest (Figure 1.1) and occasionally at the abdomen (e.g. Björnsdóttir *et al.*, 2006; van den Boom *et al.*, 2008). Although age at onset of IBH varies greatly (Anderson *et al.*, 1988; Eriksson *et al.*, 2008; van den Boom *et al.*, 2008), average age at onset is between 2 and 4 years-of-age. Severely affected horses are difficult to ride or show due to discomfort and disfiguration and are sometimes euthanized and therefore impairs animal welfare (Fadok and Greiner, 1990; Gortel, 1998); their commercial value is reduced and owners encounter extra costs in an attempt to control the itch and thereby minimize self-inflicted trauma

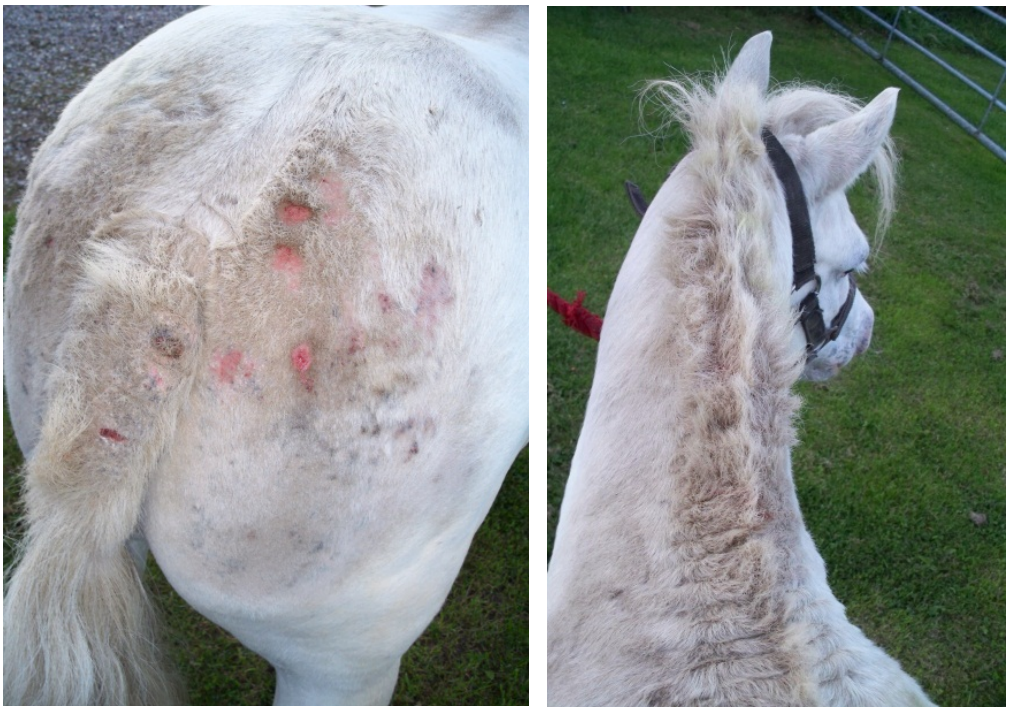

Figure 1.1 Severe clinical symptoms of insect bite hypersensitivity in an Appaloosa pony.

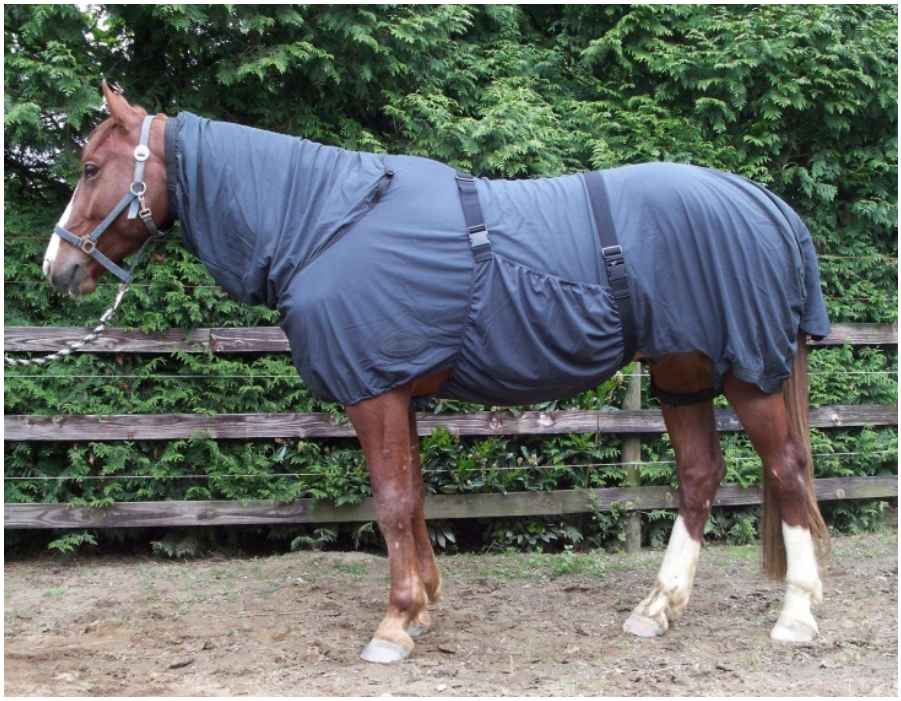

Figure 1.2 A protective rug used as preventive measure against insect bite hypersensitivity.

(Gortel, 1998). Preventive measures against IBH often aim at reducing exposure to *Culicoides* spp. through stabling, use of protective rugs (Figure 1.2) or insect repellents (Gortel, 1998; Meiswinkel *et al.*, 2000; Papadopoulos *et al.*, 2010). At present, fully effective measures to prevent or cure IBH are unavailable. IBH is multifactorial in origin and involves environmental and genetic factors (Unkel *et al.*, 1987; Marti *et al.* 1992; Eder *et al.*, 2001). Environmental factors like geographic region and climate are often linked with *Culicoides* spp. activity or density (e.g. Takken *et al.*, 2008) and therefore contribute to IBH prevalence. Several studies suggested a genetic predisposition to IBH (Riek, 1953; Anderson *et al.*, 1988), but actual genetic research on IBH is limited.

1.1.2 Etiology

IBH is an immediate or type I hypersensitivity reaction to *Culicoides* spp. and sometimes black flies (*Simulium* spp.) or other insects (e.g. Fadok and Greiner, 1990; Marti *et al.*, 1999; Baselgia *et al.*, 2006). More specifically, salivary proteins are presumed to induce the hypersensitivity (Wilson *et al.*, 2008; Langner *et al.*, 2009; Russell *et al.*, 2009). Using a modified Prausnitz-Küstner experiment, Wagner *et al.* (2006) demonstrated that indeed IgE mediated type I hypersensitivity and *Culicoides* specific IgE antibodies contribute to IBH in horses. A delayed type or type IV hypersensitivity has been demonstrated in severely affected horses indicating

the involvement of allergen specific T cells (Fadok and Greiner, 1990; Langner *et al.*, 2008; Langer *et al.*, 2009; Sloet van Oldruitenborgh-Oosterbaan *et al.*, 2009).
Allergies develop slowly: many immunological processes occur before clinical symptoms can be observed. *Culicoides* specific IgE antibodies are produced in allergic individuals and sensitize mast cells. Subsequent contact of sensitized individuals with the otherwise harmless salivary proteins of *Culicoides* spp., results in an immediate release of inflammatory mediators and the development of clinical symptoms (Wagner *et al.*, 2008). However, sensitization is also observed in clinically healthy individuals (Kobelt, 2001; Wagner *et al.*, 2008). Properties of the allergens (e.g. form and amount) may be critical in arousing the hypersensitivity reaction and thereby clinical symptoms (Kobelt, 2001; Langner *et al.*, 2008). Sensitization in clinically healthy individuals might predict prospective development of symptoms in these individuals (Wagner *et al.*, 2008) and also suggests that specific regulatory mechanisms – that might be different or even absent in affected individuals – control the reaction to the allergens (Kobelt, 2001; Wagner *et al.*, 2008).

1.1.3 Prevalence

IBH prevalence in various horse breeds as observed in many countries varies greatly (Table 1.1) and is considered a serious problem worldwide. Differences in reported prevalence might for instance be due to differences in environment related to *Culicoides* spp. activity or density and differences between horse breeds related to genetic predisposition for IBH (e.g. Littlewood, 1998). Also, the way of data collection varies between studies (Table 1.1). Some reported prevalences were estimated using an unselected sample of the population thereby representing population means (van Grevenhof *et al.*, 2007), whereas others were based on questionnaire data and might be overestimated as owners of affected horses are more likely to participate (e.g. Anderson *et al.*, 1988; van den Boom *et al.*, 2008).
Culicoides spp. are absent in Iceland, where IBH is not observed (Marti *et al.*, 2008). Prevalence in Icelandic horses imported from Iceland was significantly higher than prevalence in Icelandic horses born in Europe (Table 1.1) (Broström *et al.*, 1987; Halldórdsóttir and Larsen, 1991). The difference in prevalence might be caused by both genetic and environmental factors. However, Swedish-born Icelandic horses and Icelandic horses imported from Iceland originated from a similar genetic background (Broström *et al.*, 1987). Increased IBH prevalence in Icelandic horses imported from Iceland is not clearly understood, but is suggested to be related to lack of exposure to *Culicoides* spp. before export and increased environmental pressure after export (Broström *et al.*, 1987; Björnsdóttir *et al.*, 2006).

Table 1.1 Overview of reported IBH prevalence (%) in various horse populations worldwide including number of investigated horses and data collection.

Country	Horse population	n	Data collection	Prevalence	Reference
	Various breeds				
British Columbia	Various breeds[a]	209	Questionnaire, telephone interview	25.8%	Anderson *et al.* (1988)
Israel	Various breeds[b]	723	Questionnaire, discussion with owners	21.8%	Braverman *et al.* (1983)
Israel	Various breeds[c]	408	Questionnaire, IBH confirmed by veterinarian	27.9%	Steinman *et al.* (2003)
Japan	Unknown[d]	48,736	61 veterinary clinics, Hokkaido region	4.4%	Nakamura *et al.* (1956)
the Netherlands	Various breeds[e]	781	Internet-based questionnaire	56.0%	van den Boom *et al.* (2008)
	Specific breeds				
the Netherlands	Friesian horses	2,824	Routine inspections, inspectors trained by veterinarian	18.1%	van Grevenhof *et al.* (2007)
the Netherlands	Shetland ponies	3,284	Routine inspections, inspectors trained by veterinarian	8.1%	van Grevenhof *et al.* (2007)
Belgium	Warmblood horses	1,406	Questionnaire, during horse competitions	9.8%	Peeters *et al.* (2010)
Britain	Shire horses	1,088	Questionnaire, 20% of total population	11.6%	Littlewood (1998)
Germany	Shire horses	77	Questionnaire, 40% of total population	37.7%	Littlewood (1998)
Czech Republic	Old Kladruber horses	155	Clinical examination, grey variety	31.0%	Hofmanová *et al.* (2008)

Table 1.1 continued

Country	Horse population	n	Data collection	Prevalence	Reference
	Icelandic horses				
Norway	Imported from Iceland[f]	255	Questionnaire, members of NIHF[g]	26.9%	Halldórdsóttir and Larsen (1991)
Sweden	Imported from Iceland[f]	279	Questionnaire, clinical examination	26.2%	Broström *et al.* (1987)
Germany, Denmark, Sweden	Imported from Iceland	330	Cross sectional study, IBH confirmed by veterinarian	34.5%	Björnsdóttir *et al.* (2006)
Norway	Norwegian-born[f]	197	Questionnaire, members of NIHF[g]	8.2%	Halldórdsóttir and Larsen (1991)
Sweden	Swedish-born[f]	224	Questionnaire, clinical examination	6.7%	Broström *et al.* (1987)
Sweden	Swedish-born	1,250	Internet-based questionnaire, offspring 33 sires	8.0%	Eriksson *et al.* (2008)
Germany	German-born	984	Cross sectional study, offspring of 36 sires	18.1%	Unkel *et al.* (1987)

[a]Thoroughbred, Quarter, Morgan, Arabian and Appaloosa horses. No significant difference in prevalence between breeds.
[b]Thoroughbred, Arabian horses, ponies, local breeds and crossbreds. Ponies were significantly more often affected.
[c]Local breed, Arabian, Thoroughbred, Warmblood and Quarter horses, ponies. Warmblood horses were significantly less often affected.
[d]Unknown from which breeds the horses originated.
[e]Friesian, Haflinger, Fjord, Tinker, Coldblood, Warmblood and Icelandic horses, Welsh, New Forest and Shetland ponies, other breeds. Warmblood horses were significantly less often affected than Friesian horses and various coldblood and pony breeds.
[f]Observed prevalence in Icelandic horses imported from Iceland was significantly higher than in Swedish-born or Norwegian-born Icelandic horses.
[g]NIHF = Norwegian Icelandic Horse Association. In total 282 owners responded with information on 391, both Icelandic horses imported from Iceland and Norwegian-born Icelandic horses.

1.1.4 *Culicoides* spp.

Culicoides spp. (Figure 1.3) are the most important insects inducing IBH (e.g. Riek, 1954; Fadok and Greiner, 1990; Marti *et al.*, 1999; Baselgia *et al.*, 2006) and are also a vector of for instance bluetongue virus and African horse sickness. *Culicoides* spp. are several millimeters in length and many sub-species (e.g. *obsoletus*, *nubeculosus*, *punctatus*) have been identified (Beuk, 2002). Female *Culicoides* spp. feed on blood that is needed for egg production, while males feed on nectar. Species causing IBH differ between countries; *C. obsoletus* was identified as causal agent in British Columbia and the Netherlands (Anderson *et al.*, 1991; van der Rijt *et al.*, 2008; Sloet van Oldruitenborgh-Oosterbaan *et al.*, 2009), while *C. imicola* was identified as causal agent in Israel (Braverman *et al.*, 1983; Braverman, 1988). *C. nubeculosus* is widespread in Germany (Kobelt, 2001), while *C. sonorensis* is observed frequently in North America (Langner *et al.*, 2009). *Culicoides* spp. are weak fliers and often remain within a short distance of their breeding site. *Culicoides* spp. prefer specific weather conditions like low wind speed, no rainfall or direct sunlight (e.g. Anderson *et al.*, 1993a; Meiswinkel *et al.*, 2000; Takken *et al.*, 2008). Association between temperature and *Culicoides* activity was unclear, although many *Culicoides* spp. were caught during periods of warm weather.

Figure 1.3 Female *Culicoides obsoletus*, partly blood-fed, identified on size and wing pattern according to Campbell and Pelham-Clinton (1960). Photograph kindly provided by ir. N.M.A. van der Meide.

1.1.5 Diagnosis

Serological, cellular and intradermal tests are developed to diagnose IBH or even discriminate sensitized (asymptotic) horses and healthy controls (Marti *et al.*, 1999; Baselgia *et al.*, 2006; Frey *et al.*, 2008; Langner *et al.*, 2008; Wagner *et al.*, 2008; Sloet van Oldruitenborgh-Oosterbaan *et al.*, 2009). Their suitability as a diagnostic test differed (e.g. Langner *et al.*, 2008) and specific drawbacks were reported. For instance, the cellular antigen simulation test (Baselgia *et al.*, 2006) proofed to have a high specificity and good sensitivity and was able to identify sensitized horses. However, test results significantly differed between month of testing and fresh blood samples were needed as sulphidoleukotriene release that defined the test result was already impaired in 24h old samples (Marti *et al.*, 1999; Baselgia *et al.*, 2006). Compared to *in vitro* tests, *in vivo* tests like intradermal testing are expected to be time- and cost-consuming, are more invasive (Wagner *et al.*, 2008) and might even sensitize individuals (Herbst *et al.*, 1993). There is no consensus concerning cross-reactivity of *Culicoides* spp. in literature (Anderson *et al.*, 1993b; Langner *et al.*, 2008; Sloet van Oldruitenborgh-Oosterbaan *et al.*, 2009). Therefore, it is inconclusive whether native *Culicoides* spp. need to be used for testing or whether exotic *Culicoides* spp. are suitable as well. Observation of clinical symptoms combined with medical history of the horse is still the gold standard to diagnose IBH (Baselgia *et al.*, 2006; Langner *et al.*, 2008), while results from *in vitro* or *in vivo* tests can contribute to the accuracy of diagnosis (Langer *et al.*, 2008; Sloet van Oldruitenborgh-Oosterbaan *et al.*, 2009).

1.1.6 Selection against insect bite hypersensitivity

Genetic variance

Selection and breeding to reduce IBH prevalence can only be successful if genetic variance for IBH sensitivity exists within a population. Several studies suggested a genetic predisposition to IBH because horses from specific families were more often affected than horses from other families (Riek, 1953; Braverman *et al.*, 1983; Anderson *et al.*, 1988; Gerber, 1989; Littlewood, 1998; van Grevenhof *et al.*, 2007). Also, Marti *et al.* (1992) reported that only horses descending from a particular sire were affected whereas other horses on the same stud and from similar age but unrelated to the particular sire were unaffected. Studies that actually estimated genetic variance and thereby the heritability for IBH are limited. A polygenic inheritance of IBH is expected (Marti *et al.*, 2008), as Unkel *et al.* (1987) rejected a monogenic inheritance of IBH through segregation analysis in 984 German-born Icelandic horses. Horses descended from 36 sires and 362 dams. Heritability of IBH

status (present/absent) on the observed scale was 0.08 (se = 0.07), using a linear sire model. Björnsdóttir *et al.* (2006) investigated phenotypes and pedigree of 330 Icelandic horses imported from Iceland and concluded that IBH heritability did not significantly differ from zero. However, a genetic predisposition was not rejected, as data were considered to be too small to estimate a heritability but successfully identified risk factors for IBH.

Candidate genes

Candidate gene research on IBH is limited. Marti *et al.* (1992) studied 304 Swiss Warmblood paternal half-sibs sired by 6 sires. Only 3 sires had affected horses among their offspring. Genetic predisposition to IBH was associated with equine leucocyte antigen class II specificity W23. Marti *et al.* (1992) concluded that genes of the equine leucocyte antigen, or major histocompatibility complex, as well as other genes contribute to IBH genetic variance. Serological research on 303 Icelandic horses imported from Iceland revealed differences in distribution of leucocyte antigen class II specificities between affected and unaffected horses (Halldórsdóttir *et al.*, 1991). Microsatellite HMS01 on chromosome 15 was significantly associated with IBH (Marti *et al.*, 2005 in Chowdhary and Raudsepp, 2008). Kolm *et al.* (2006) examined mRNA expression of *IL1β* (interleukin 1, beta) and *IL1RA* (interleukin 1, receptor antagonist) in the skin of affected and unaffected horses before and after intradermal injection with *C. nubeculosus* extract. They found no significant difference in expression of *IL1RA* between affected and unaffected horses and neither before or after challenge. However, *IL1β* (interleukin 1, beta) transcription was significantly upregulated from basal levels after intradermal injection of *C. nubeculosus* in affected horses only.

Selection and breeding

Unkel *et al.* (1987) showed that IBH prevalence in German-born Icelandic horses (18.1%) could be reduced with 3.6% per generation when affected horses were excluded from breeding (i.e. mass selection) assuming a heritability of 0.2, which was considered an upper limit. Reducing prevalence from 18.1% to 8% using a more realistic assumption (h^2 = 0.1) would take roughly 8 generations (Unkel *et al.*, 1987). Unkel *et al.* (1987) considered the reduction in IBH prevalence in their calculations, as the rate of genetic improvement depends on prevalence and therefore reduces in subsequent generations. Although selection based on own performance will reduce IBH prevalence, it will take many generations to realize a considerable reduction (e.g. Unkel *et al.*, 1987). Selection against IBH by studbooks is limited. Several studbooks (e.g. Dutch Shetland Pony Studbook and Royal Friesian

Horse Studbook) report that IBH is incorporated in their selection decisions. Regulations of the Dutch Shetland Pony Studbook state that "clinical symptoms of IBH should not be observed" (http://www.nsps.nl), yet affected studbook mares are used in breeding. Phenotypic information on IBH is often scarce and highly depends on exposure to *Culicoides* spp. Moreover, estimated breeding values are unavailable.

1.2 Aim and outline of this thesis

The aim of the research described in this thesis was to increase our understanding of the genetic background of IBH to be able to reduce IBH prevalence in horse populations in the Netherlands through selection and breeding.

An increased knowledge of risk factors associated with IBH is necessary to demonstrate how these factors interfere with the genotype. Therefore, in chapter 2, risk factors affecting occurrence of IBH in both Friesian horse and Shetland pony mares in the Netherlands were identified and quantified. In total 3,453 Friesian horse mares and 7,608 Shetland pony mares were visually scored for IBH clinical symptoms by inspectors during obligatory foal inspections in several years.

Genetic variance is essential for selection and breeding against any disorder. Genetic variance and thereby heritability of IBH in Shetland pony mares and Friesian horse mares were estimated and results are presented in chapter 3 and 4, respectively. When *Culicoides* spp. are present, sensitive horses will develop IBH clinical symptoms year after year. To investigate whether repeated observations on IBH have potential to increase accuracy of breeding values, repeatability of IBH was estimated in both breeds as well. Environments and events in early life are expected to contribute considerably to sensitivity to allergies. In chapter 4, a maternal effect, as part of early life environment, was estimated in Friesian horse mares to investigate whether the dam affected the development of IBH in offspring by being part of their rearing environment.

Knowledge on genes contributing to and genomic regions associated with IBH sensitivity will increase our understanding of its biology and will enable the development of more efficient prevention, therapy and selection against IBH. In chapter 5 and 6, genomic regions associated with IBH in Shetland pony mares and Icelandic horses in the Netherlands were identified and quantified using genome-wide association studies. Data were obtained through a matched case-control design. A protocol for data collection was developed to prevent population stratification and thereby false positive results. In chapter 5, genotypes (50k) from 188 Shetland pony mares were analyzed using single-SNP logistic regression. In

chapter 6, genotypes (70k) from 200 Shetland pony mares and 146 Icelandic horses were analyzed using a threshold model and Bayesian variable selection method Bayes-C that fitted all SNP simultaneously.
Chapter 7, the general discussion, explored possibilities to reduce IBH prevalence in horse populations within the Netherlands through breeding against IBH. Several breeding strategies were simulated using own performance, progeny testing or genomic selection. Genetic gain of simulated strategies were compared and implications concerning practical horse breeding were discussed. Also, various phenotypes of IBH like clinical symptoms and results from *in vitro* and *in vivo* tests including associated environmental factors were discussed.

Research described in this thesis is part of a large-scale project in the Netherlands focusing on immunological and genetic aspects of IBH. Researchers from both Wageningen University and University Utrecht cooperated in this project. Together, they worked on the development of improved diagnosis, efficient intervention strategies – related to risk factors, genetic predisposition and desensitization – and therapeutic approaches that effectively reduce IBH prevalence in horse populations in the Netherlands. The IBH project was financially supported by technology foundation STW, the Dutch federation of horse breeding Vereniging Koepel Fokkerij and the pharmaceutical company Artu Biologicals, part of ALK-Abelló.

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2

Risk factors for insect bite hypersensitivity in Friesian horses and Shetland ponies in the Netherlands

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Abstract

Insect bite hypersensitivity (IBH) is an equine skin allergy caused by bites of *Culicoides* spp. and impacts on the welfare of affected horses. The aim of this study was to identify and quantify risk factors for IBH. Data from 3,453 Friesian horse mares and 7,074 Shetland pony mares scored for IBH by inspectors during obligatory foal inspections were analyzed using breed-specific multivariable logistic regression models. The combined effect of month and year of scoring, province and inspector were significantly associated with IBH in both breeds. In Shetland pony mares, withers height and coat colour were also significantly associated with IBH, while body condition had a nearly significant effect. The outcomes from this study on risk factors might contribute to the development of more efficient measures to reduce prevalence of IBH.

Key words: body condition, horse, insect bite hypersensitivity, risk factors, summer eczema

2.1 Introduction

Insect bite hypersensitivity (IBH) is a common allergic skin disease found in many horse breeds worldwide and is caused by bites of *Culicoides* spp. (Braverman *et al.*, 1983). IBH seriously reduces the welfare of affected horses. The aetiology of IBH is multifactorial and involves both environmental and genetic factors (Schurink *et al.*, 2011). Geographic region, year, breed, gender, age and coat colour of horses have been suggested as potential risk factors in studies using data from questionnaires (Braverman *et al.*, 1983; Halldórdsóttir and Larsen, 1991; van den Boom *et al.*, 2008). However, the literature reveals contradicting results, which can partially be attributed to the small size of datasets used. In humans, many studies show that obesity is a risk factor for allergies (Hersoug and Linneberg, 2007) but, to the best of our knowledge, body condition (i.e. the amount of fat stored in a body) has not been investigated as a potential risk factor for IBH in horses. The aim of the current study was to identify and quantify risk factors related to IBH in two horse breeds in the Netherlands.

2.2 Materials and methods

Friesian horse (FH) and Shetland pony mares (SP) were visually scored for IBH once a year (Table 2.1 and 2.2) during obligatory foal inspections, which enabled the collection of many records. Scoring of IBH clinical symptoms was made as uniform as possible during a training session. After editing, the dataset contained 3,763 IBH observations from 3,453 FH and 10,079 IBH observations from 7,074 SP. For each breed potential risk factors were analyzed separately by applying a backward procedure and logistic regression with 'animal' as repeated effect to account for multiple observations per mare using the GENMOD procedure in SAS® 9.2 (SAS Institute Inc. Cary, NC).

2.3 Results and discussion

A large dataset containing unselected data was analysed since all mares with foals entering the studbook were scored for IBH. Prevalence within FH was 18.2% and within SP 7.5%. Breed-specific analyses were performed as breed was completely confounded with year of scoring and inspector. Scoring mares on symptoms by inspectors during foal inspections might not be the most accurate method (i.e. inspector was significantly related to IBH outcome), but scoring clinical symptoms is still the gold-standard. Inspector and region in SP and inspector and year in FH were partly confounded and as a consequence their effects could not be separated.

Table 2.1 Multivariable logistic regression outcomes of risk factors (P <0.05) for insect bite hypersensitivity in Friesian horse mares (n = 3,453) in the Netherlands[a].

Variable	n	Prevalence	OR (95% CI)[b]	P-value
Inspector[c]	13-1,361	14.7-37.5	1.0-13.2	<0.0001
Province[d]				0.0058
Drenthe	245	20.8	1.1 (0.8-1.6)	
Flevoland & Noord-Holland	119	9.2	0.4 (0.2-0.8)	
Friesland	2,425	17.0	1.0	
Gelderland	255	22.0	1.3 (0.9-1.9)	
Groningen	191	22.5	1.4 (1.0-2.0)	
Limburg & Noord-Brabant	165	20.6	1.3 (0.8-2.0)	
Overijssel	207	24.2	1.4 (1.0-2.0)	
Utrecht	53	20.8	1.1 (0.6-2.2)	
Zeeland & Zuid-Holland	103	14.6	0.8 (0.5-1.5)	
Month × year				0.0003
June 2004	167	15.0	1.0	
July 2004	806	15.5	1.2 (0.7-2.0)	
August 2004	957	18.9	1.3 (0.8-2.2)	
September 2004	661	19.5	1.5 (0.9-2.4)	
October 2004	160	31.3	2.0 (1.0-3.9)	
June 2008	40	12.5	4.2 (0.9-19.9)	
July 2008	244	16.0	5.2 (1.4-19.8)	
August 2008	404	18.3	6.4 (1.9-21.3)	
September 2008	267	19.9	9.7 (2.6-35.6)	
October 2008	57	5.3	NE[e]	

[a]Correlation between repeated observations was 0.22. Age at observation modeled as a 2nd order polynomial had a nearly significantly effect on insect bite hypersensitivity (P linear term = 0.0542 and P quadratic term = 0.0292).
[b]Odds ratio (95% confidence interval).
[c]12 inspectors scored Friesian horse mares. Range of number of observations, prevalence and odds ratio per inspector are presented.
[d]The province of Friesland was set as the reference province to enable direct comparison with results from Shetland pony mare data.
[e]NE, not estimable due to confounding with inspector 12.

Factors included in the final multivariable model for FH are presented in Table 2.1 and for SP in Table 2.2. Despite a significant higher prevalence in mares kept stabled in both breeds, housing system was not included in the model as stabling of horses was considered a consequence of IBH instead of a causative effect (van Grevenhof *et al.*, 2007). Stabling as a preventive method is considered most effective in closed stables (Meiswinkel *et al.*, 2000), especially during sunset when

Culicoides spp. were found to be most active. Provinces within the Netherlands and month and year of scoring were significantly associated with IBH in SP and FH. Identified low- and high-risk regions for both breeds largely overlap (Table 2.1 and 2.2) and were in agreement with previous studies (van Grevenhof *et al.*, 2007; van den Boom *et al.*, 2008). Regions containing soils of clay with heather and woody vegetation and low annual rainfall, few cold days and many warm days per year were associated with increased prevalence (van Grevenhof *et al.*, 2007). Regions with low prevalence were found along the coast line, related to high wind speeds, which suppresses *Culicoides* spp. density (Meiswinkel *et al.*, 2000). Shelter and moisture at pasture significantly coincided with increased IBH prevalence in Icelandic horses (Björnsdóttir *et al.*, 2006). Annual and monthly variations in *Culicoides* spp. population density were related to changes in environmental

Table 2.2 Multivariable logistic regression outcomes of risk factors (P <0.05) for insect bite hypersensitivity in Shetland pony mares (n = 7,074) in the Netherlands[a].

Variable	n	Prevalence	OR (95% CI)[b]	P-value
Inspector[c]	144-1,400	1.7-23.3	1.0-31.0	<0.0001
WHC[d]				0.0297
Mini	2,876	5.7	1.1 (0.8-1.4)	
Small	2,231	6.2	1.0	
Middle	2,439	8.7	1.4 (1.1-1.7)	
Tall	2,533	8.6	1.4 (1.1-1.8)	
Coat colour				0.0172
Bay	626	4.5	1.0	
Black	4,131	8.6	1.6 (1.0-2.4)	
Black paint	332	9.0	1.9 (1.0-3.3)	
Chestnut	3,643	6.5	1.1 (0.7-1.7)	
Chestnut paint	562	6.9	1.3 (0.7-2.2)	
Other	785	5.6	1.3 (0.7-2.1)	
Province				0.0013
Drenthe	738	12.7	3.6 (1.5-8.6)	
Flevoland & Noord-Holland	61	6.6	3.6 (0.7-20.3)	
Friesland	408	2.7	1.0	
Gelderland	3,200	9.8	5.0 (1.5-16.2)	
Groningen	326	11.0	4.4 (2.2-9.0)	
Limburg	1,248	2.4	9.2 (2.5-34.0)	
Noord-Brabant	2,196	7.6	5.8 (1.7-20.0)	
Overijssel	1,302	3.9	2.8 (1.0-7.9)	
Utrecht	122	3.3	7.3 (1.5-35.2)	
Zeeland & Zuid-Holland	478	4.0	2.1 (0.5-9.6)	

Table 2.2 continued

Variable	n	Prevalence	OR (95% CI)[b]	P-value
Month × year				<0.0001
2003[e]	2,946	8.3	9.3 (2.2-39.3)	
June & July 2005	110	7.3	10.1 (2.1-48.8)	
August & September 2005	1,199	8.9	12.8 (3.0-54.5)	
October & November 2005	450	9.6	14.0 (3.2-60.4)	
June & July 2006	414	9.2	12.2 (2.8-53.5)	
August & September 2006	1,921	8.2	6.7 (1.6-28.3)	
October & November 2006	432	6.0	7.5 (1.7-33.3)	
June & July 2009	672	9.1	8.3 (2.0-34.9)	
August & September 2009	1,599	2.6	2.7 (0.6-11.1)	
October & November 2009	336	0.9	1.0	

[a]Correlation between repeated observations was 0.14. Age at observation did not significantly affect insect bite hypersensitivity (P >0.05).
[b]Odds ratio (95% confidence interval).
[c]17 inspectors scored Shetland pony mares. Range of number of observations, prevalence and odds ratio per inspector are presented.
[d]WHC, withers height category: mini ≤86 cm, small 87 to 92 cm, middle 93 to 98 cm and tall 99 to 107 cm.
[e]Month of scoring not registered in 2003.

conditions (Takken *et al.*, 2008). The preferences of *Culicoides* spp. for specific environmental conditions will lead to differences in exposure of horses to *Culicoides* spp., resulting in regional, annual and even monthly varying IBH prevalence.

Age at observation did not significantly affect IBH. Published results concerning an association between age and IBH are controversial, although an increased risk with age is expected to be related to longer exposure to factors that enhance the development of IBH and not necessarily due to an increased susceptibility to IBH (Halldórdsóttir and Larsen, 1991). In SP, withers height category (WHC) and coat colour were significantly related to IBH (Table 2.2). Middle height and tall mares and black paint mares had increased odds to show symptoms, compared to small and bay mares. We hypothesize that WHC is not an effect in itself (e.g. genetic effect, as rejected by Schurink *et al.*, 2009), but was related to different selection decisions made by owners of various WHC. Coat colours might differ in attractiveness for *Culicoides* spp., but the effect of specific coat colours on IBH differed between studies (Braverman *et al.*, 1983) and many studies did not find an effect (Halldórdsóttir and Larsen, 1991; van Grevenhof *et al.*, 2007). Due to

Table 2.3 Logistic regression outcomes of body condition score (i.e. the amount of fat stored in a body) using a multivariable model for insect bite hypersensitivity in Shetland pony mares in the Netherlands[a].

Variable	n	Prevalence	OR (95% CI)[b]	P-value
Body condition score				0.063
Low	146	6.2	1.5 (0.7-3.2)	
Normal	2,535	3.7	1.0	
High	128	9.4	2.3 (1.2-4.5)	

[a]Body condition was scored simultaneously with insect bite hypersensitivity in 2009 on a subset of the investigated Shetland pony mares (n = 2,809). Other factors significantly affecting insect bite hypersensitivity in the multivariable model on this subset were inspector (P <0.0001) and combined effect of month and year of scoring (P <0.0001).
[b]Odds ratio (95% confidence interval).

confounding between WHC and coat colour, these effects could not be separated in this study.

In SP, mares with either low or high body condition score (BCS) showed increased odds to present IBH symptoms compared to mares with normal BCS (Table 2.3). BCS measured during studbook inspections (i.e. separate from IBH recording) was not significantly associated with IBH (data not shown). Hersoug and Linneberg (2007) hypothesized that the risk of allergies was increased in obese individuals at least partly due to immunological changes, since white adipose tissue secrete more adipokines and cytokines, resulting in a decreased immunological tolerance to antigens and skewing towards a Th2-biased immune response. We could not confirm the hypothesis that body condition was a potential risk factor for IBH, although the odds of IBH was significantly increased in horses with high BCS compared to horses with normal BCS (OR = 2.3). Ideally, a cohort study should be performed to better investigate and verify the effect of body condition on (the development of) IBH.

In summary, the combined effect of month and year of IBH scoring, inspector and region were risk factors for IBH in both FH and SP in the Netherlands. WHC and coat colour were also significantly related to IBH in SP, while BCS had a nearly overall significant effect. Our findings and results from other research suggested that exposure to *Culicoides* spp. should be reduced to decrease or even prevent IBH clinical symptoms. Sensitive horses could be moved to low-risk habitats or the current habitat could be adapted to reduce exposure to *Culicoides* spp. IBH prevalence is increased in horses with specific coat colours, though identified high-risk coat colours differ between studies. Maintaining a normal body condition would help to minimize the odds of having IBH.

2.4 Acknowledgements

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3

Heritability and repeatability of insect bite hypersensitivity in Dutch Shetland breeding mares

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Abstract

Insect bite hypersensitivity (IBH) is a seasonal recurrent allergic reaction of horses to the bites of certain *Culicoides* spp. and is found throughout the world. The aim of our study was to estimate the heritability and repeatability of IBH in the Dutch Shetland pony population. A total of 7,924 IBH scores on 6,073 mares were collected during foal inspections in 2003, 2005, and 2006. Mares were scored for clinical symptoms of IBH from June until February by 16 inspectors. Of all mares, 74.4% (n = 4,520) had a single observation, 20.7% (n = 1,255) had 2 observations, and 4.9% (n = 298) had 3 observations in different years. The overall mean IBH prevalence was 8.8%. Heritability was 0.08 (se = 0.02) on the observed binary scale and 0.24 (se = 0.06) on the underlying continuous scale. Repeatability was 0.30 (se = 0.02) and indicates that including repeated observations of the clinical symptoms of IBH will improve the accuracy of breeding values for IBH. We conclude that IBH, based on clinical symptoms, is a heritable trait in the Dutch Shetland pony population. Therefore, the IBH prevalence in this population can be lowered by selection.

Key words: heritability, horse, insect bite hypersensitivity, repeatability

3.1 Introduction

Insect bite hypersensitivity (IBH) is a seasonal recurrent allergic reaction of horses to the bites of certain *Culicoides* spp. (Riek, 1954). The allergic reaction causes intense pruritus, which results in self-inflicted trauma; open wounds and secondary infections may follow. Therefore, the welfare of affected horses is seriously reduced. The commercial value of affected horses is also reduced because of disfiguration (Broström *et al.*, 1987; Fadok and Greiner, 1990). Due to extreme discomfort, affected horses are often unsuitable for riding or showing purposes (Gortel, 1998). There is no currently available effective treatment for or prevention against IBH (Anderson *et al.*, 1996; Friberg and Logas, 1999; Pilsworth and Knottenbelt, 2004).

The etiology of IBH is multifactorial in origin and involves environmental and genetic factors (Lange, 2004; Björnsdóttir *et al.*, 2006; Grandinson *et al.*, 2006). Although warm- and cold-blooded horses of various breeds worldwide are affected (Anderson *et al.*, 1988; Littlewood, 1998; Steinman *et al.*, 2003), heritabilities of IBH have been estimated only in small populations of Icelandic horses (Unkel *et al.*, 1987; Lange, 2004; Grandinson *et al.*, 2006). Therefore, no heritability estimates in breeds other than Icelandic horses are available in the scientific literature.

Sensitization to an allergen is fundamental for allergy development (Holgate and Polosa, 2008). Initial sensitization occurs at a young age, and subsequent exposure to the otherwise-harmless allergen rouses the allergic reaction (Galli, 2000; Holgate and Polosa, 2008). Once affected, horses develop IBH year after year (Anderson *et al.*, 1991); therefore, high repeatability of allergies should be expected even if no estimates of IBH repeatability are available in the scientific literature.

The aim of our study was to estimate the heritability and repeatability of IBH in Dutch Shetland breeding mares to investigate the potential of breeding against IBH and to gain information on the potential of repeated observations on IBH to increase accuracy of selection. Heritability and repeatability were estimated by analyzing phenotypic observations on clinical symptoms of IBH collected during 3 years.

3.2 Materials and methods

Animal Care and Use Committee approval was not required for our study because the collection of phenotypes was a noninvasive method additional to the routine foal inspections.

Table 3.1 Data description and observed insect bite hypersensitivity (IBH) prevalence per year and overall.

Variable	Year of scoring			Overall
	2003	2005	2006	
Observations, n (%)[a]	3,063 (38.6)	1,948 (24.6)	2,913 (36.8)	7,924
Observed prevalence, %	8.6	9.5	8.5	8.8
Mean age, yr (range)	8.1 (3–24)	7.8 (3–22)	8.2 (3–25)	8.1 (3–25)
Housing system, %				
Partly stabled	ND[b]	2.0	2.2	2.1
Kept on pasture	ND	98.0	97.8	97.9
WHC[c], %				
Mini	31.4	28.9	26.4	29.0
Small	20.6	21.9	22.5	21.6
Middle	23.1	23.7	25.2	24.0
Tall	24.9	25.5	25.9	25.4

[a]Percentage of total number of observations.
[b]ND = no data.
[c]WHC = withers height category: mini = ≤ 86 cm; small = 87 to 92 cm; middle = 93 t0 98 cm; and tall = 99 t0 107 cm.

3.2.1 Data

Data on IBH were collected on Dutch Shetland breeding mares from 58 of 90 (64%) regions across the Netherlands, based on 2-digit postal codes, constituting a representative sample of the Dutch Shetland pony breed. The data were collected during foal inspections in 2003, 2005, and 2006 (Table 3.1). During foal inspections, newborn foals are judged at home by inspectors for studbook entrance. In 2003, the date of scoring was not registered.

Mares were, on average, 8.1 yr of age (sd = 4.1) and were either kept on pasture or partly stabled (Table 3.1). Mares were grouped by the Dutch Shetland Pony Studbook into 4 categories according to their height at the withers in centimeters. The categories were mini, ≤ 86 cm; small, 87 to 92 cm; middle, 93 to 98 cm; and tall, 99 to 107 cm (Table 3.1).

3.2.2 Trait definition of IBH

In each year, mares were scored for IBH from June until February by 16 inspectors. The IBH scores from December, January, and February (n = 127) were not used, because clinical symptoms of IBH regress during winter when *Culicoides* spp. are usually absent (Anderson *et al.*, 1988). All inspectors were trained by a veterinarian to score clinical symptoms of IBH by visual observation of occurrence of lesions on

IBH-sensitive locations at the body, such as the mane, the base of the tail, and the ventral midline.

The IBH status of mares was scored in 3 categories: 0 = absence of clinical symptoms, 1 = dubious or mild clinical symptoms (hair loss), and 2 = clear clinical symptoms (at least bald spots and possible open wounds). Similar IBH scoring methods were used by other researchers (e.g. Broström *et al.*, 1987; Steinman *et al.*, 2003). Previous results on a subset of our data indicated that IBH categories 1 and 2 were more similar than IBH categories 0 and 1 (van Grevenhof *et al.*, 2007). Therefore, IBH categories 1 and 2 were grouped together, and resulting IBH scores were analyzed as a binary trait.

3.2.3 Pedigree information

For the genetic analysis, available pedigree information over 4 generations was considered. The pedigree information was provided by the Dutch Shetland Pony Studbook. Mares descended from 984 sires and 4,455 dams. In total, 0.1% (n = 8) of the mares were descended from an unknown sire, 0.3% (n = 16) of the mares were descended from an unknown dam, and 0.7% (n = 44) of the mares were descended from both an unknown sire and an unknown dam. Sires had, on average, 8.0 offspring in the data, and dams had, on average, 1.2 offspring in the data.

3.2.4 Statistical analysis

Tested fixed effects

Fixed effects considered in our study were year and month of scoring, housing system, climate and habitat, coat color, withers height category, and age. Year and month of scoring might affect IBH prevalence, because the presence and activity of *Culicoides* spp. can differ between years and months (Anderson *et al.*, 1993). Many researchers (e.g. Anderson *et al.*, 1988; Björnsdóttir *et al.*, 2006) advise housing of IBH-sensitive horses to avoid contact with *Culicoides* spp. as a method of prophylaxis. Housing system was tested but a priori not included in the model, because the effect would undeservedly correct for the high IBH prevalence in horses kept stabled (van Grevenhof *et al.*, 2007). van Grevenhof *et al.* (2007) showed that IBH scores were affected by climate and habitat factors, and defined 2 explanatory index variables. The continuous variable “climate” was based on information regarding annual number of cold and warm days and annual rainfall. The class variable “habitat” was based on information regarding soil type and vegetation. For more details about climate and habitat, see van Grevenhof *et al.* (2007). The findings of van Grevenhof *et al.* (2007) confirmed their expectations,

because *Culicoides* spp. prefer certain weather (Meiswinkel *et al.*, 2000), and an association between IBH prevalence and any particular biotype was suggested by Broström *et al.* (1987). Horses with dark coat color were more often affected by IBH (Braverman *et al.*, 1983; Lange, 2004). Therefore, the attractiveness of horses with certain coat colors to *Culicoides* spp. could differ (Braverman *et al.*, 1983). Withers height category was considered, because breeding in the Dutch Shetland pony population is practiced within the categories. Age of onset of clinical symptoms of IBH varies widely and there is no fixed threshold age after which the development of clinical symptoms is no longer possible (Anderson *et al.*, 1988; Halldórdsóttir and Larsen, 1991). Some researchers (e.g. Broström *et al.*, 1987; Halldórdsóttir and Larsen, 1991) have shown that IBH prevalence increased with the age of the horse. This does not necessarily indicate an increased susceptibility to IBH in older horses because of age per se, but is thought to be a reflection of an increased risk of exposure to predisposing factors (Halldórdsóttir and Larsen, 1991).

Statistical model

Fixed effects were established using GLM (SAS Institute, Cary, NC). A linear repeatability animal model was used to estimate variance components, heritability, and repeatability of IBH, using the ASREML program (Gilmour *et al.*, 2006). The model (model 1) was

$$y_{ijklm} = \mu + yearmonth_i + b_1 \times climate_m + habitat_j + WHC_k + age_l + animal_m + pe_m + e_{ijklm},$$

where y_{ijklm} = the IBH score (0 or 1); μ = the population mean; $yearmonth_i$ = the fixed combined class effect of year and month of scoring (i = 1, 2, 3, …, 15); b_1 = the regression coefficient of the continuous covariable of climate ($climate_m$); $habitat_j$ = the fixed class effect of habitat (j = 1, 2, 3, …, 9); WHC_k = the fixed class effect of withers height category (k = 1, 2, 3, 4); age_l = the fixed class effect of age in years (l = 3, 4, 5, …, 25); $animal_m$ = the random genetic effect of the mth mare (m = 1, 2, 3, …, 6,073) with $\sim N\left(0, \mathbf{A}\sigma_a^2\right)$; pe_m = the random permanent environmental effect of the mth mare (m = 1, 2, 3, …, 6,073) with $\sim N\left(0, \mathbf{I}_{pe}\sigma_{pe}^2\right)$, and e_{ijklm} = the random residual error with $\sim N\left(0, \mathbf{I}_e\sigma_e^2\right)$; $\mathbf{I}_{pe}$ and $\mathbf{I}_e$ are identity matrices of the appropriate dimensions, and $\mathbf{A}$ is a matrix of additive genetic relationships among all Shetland ponies in our study (e.g. Lynch and Walsh, 1998).

The model was extended with a fixed class effect of Inspector (model 2) to evaluate the influence of this additional effect on heritability estimates. Model 2 was

$y_{ijklmn} = \mu + yearmonth_i + b_1 \times climate_n + habitat_j + WHC_k + age_l + inspector_m + animal_n + pe_n + e_{ijklmn}$,

where $inspector_m$ = the fixed class effect of inspector (m = 1, 2, 3, …, 16), and other components are as described previously.

3.2.5 Heritability and repeatability

The heritability on the observed binary (IBH positive or negative) scale (h^2_{obs}) was

$$h^2_{obs} = \frac{\sigma^2_a}{\sigma^2_a + \sigma^2_{pe} + \sigma^2_e},$$

where σ^2_a = the additive genetic variance, σ^2_{pe} = the permanent environmental variance, and σ^2_e = the error variance. Permanent environmental factors are those factors that cause similarity in repeated observations on the same mare. The additive genetic variance, permanent environmental variance, and error variance here together constituted the total phenotypic variance.

The heritability on the observed binary scale was transformed to an underlying continuous scale according to Dempster and Lerner (1950):

$$h^2_{und} = h^2_{obs} \frac{[p(1-p)]}{z^2},$$

where h^2_{und} = the heritability on the underlying continuous scale, h^2_{obs} = the heritability on the observed binary scale, p = the fraction of affected mares (p = 0.088), and z = the ordinate of a standard normal distribution at the threshold point corresponding to the fraction p. The heritability on the underlying scale can be interpreted as the heritability of the genetic predisposition to IBH presuming a continuous scale.

The repeatability (r) is the correlation between repeated observations within a mare; that is,

$$r = \frac{\sigma_a^2 + \sigma_{pe}^2}{\sigma_a^2 + \sigma_{pe}^2 + \sigma_e^2}$$

where all variance components are as described previously.

3.3 Results and discussion

A total of 7,924 IBH scores from 6,073 mares were analyzed. Of all mares, 74.4% (n = 4,520) had a single observation, 20.7% (n = 1,255) had 2 observations, and 4.9% (n = 298) had 3 observations in different years. In total, 694 of the 7,924 IBH scores indicated the presence of clinical symptoms; the overall mean IBH prevalence was 8.8%. The IBH prevalence was greatest in 2005 and least in 2006 (Table 3.1). The IBH status of 34.2% of the dams (n = 1,525) was known, because these dams were present as mares in the data. The observed IBH prevalence in mares descending from affected dams was greater (13.4%) than the prevalence in mares descending from unaffected dams (7.7%). The observed IBH prevalence of progeny groups per sire varied from 0 to 37%.

3.3.1 Choice of statistical model

Preliminary results showed that housing system (P = 0.94) and coat color (P = 0.56) were not significant. The effect of the month in which IBH was scored differed among years; therefore, a combined effect of year and month of scoring was included in the model (P = 0.01). Prevalence of IBH tended to increase slightly with age (P = 0.11).

From the literature it is known that there are regional differences in presence and activity of *Culicoides* spp. (e.g. Riek, 1954; Broström *et al.*, 1987; Grandinson *et al.*, 2006; van Grevenhof *et al.*, 2007). To correct for regional environmental effects that affect the presence and activity of *Culicoides* spp., climate (P = 0.005) and habitat (P < 0.0001) were included in the model instead of region based on the 2-digit postal codes in the Netherlands. Region not only expressed regional environmental effects, but also, in part, differences among inspectors, because inspectors scored regionally. Therefore, inspector and region were partly confounded.

Withers height category was highly significant (P < 0.0001). Observed IBH prevalence in mares from the mini withers height category was 5.6%, from the

small category was 7.0%, from the middle category was 9.9%, and from the tall category was 10.5%. These differences in prevalence might be caused in part by genetic differences among withers height categories, because breeding is practiced within withers height categories. Excluding withers height category from the model could result in an overestimation of the heritability, and, thereby, overestimation of genetic gain within the Shetland pony population. However, the heritability estimate was equal (data not shown) when withers height category was excluded from the model.

3.3.2 Heritability

The heritabilities of IBH estimated with the applied models were 0.08 (model 1, excluding the inspector) and 0.05 (model 2, including the inspector as a fixed effect) on the observed binary scale, and 0.24 (model 1) and 0.17 (model 2) on the underlying continuous scale (Table 3.2). Estimated heritabilities indicate that IBH, based on clinical symptoms, is a heritable trait in the Dutch Shetland pony population. Including inspector as a fixed effect in the model resulted in a decreased heritability estimate (0.05), because additive genetic variance decreased 33% when compared with additive genetic variance estimated with model 1 (Table 3.2). Including inspector as a random effect in the model resulted in an equal heritability estimate (0.05, se = 0.02). The decrease in additive genetic variance might be caused by the fact that inspectors scored regionally. Shetland pony breeding in the Netherlands is regionally organized, primarily because no artificial insemination is used. Therefore, the distribution of Shetland pony families has a regional character, such that different inspectors probably score different Shetland pony families. The partial confounding of inspectors with Shetland pony families could therefore have resulted in underestimation of the heritability when inspector was included in the model. We consider that model 1 (excluding the inspector) provides a better estimate of the heritability. To avoid a genetic effect related to inspectors, inspectors should score mares throughout the Netherlands during future data collection.

The estimated heritability in our study (0.08) is less than the heritability reported by other researchers. This might be because of differences in the observed IBH prevalence (Unkel *et al.*, 1987; Lange, 2004), breed differences (Unkel *et al.*, 1987; Lange, 2004; Grandinson *et al.*, 2006), differences in analysis methods (Unkel *et al.*, 1987), and differences in how the clinical symptoms of IBH are scored (Unkel *et al.*, 1987; Lange, 2004; Grandinson *et al.*, 2006). The clinical symptoms of IBH were scored in various ways, but heritability of binary IBH scores did not differ from heritability of IBH scores on a 0-1-2 scale in our study (data not shown). In Lange

(2004), heritability of binary IBH scores (0.36, se = 0.08) hardly differed from heritability of more detailed IBH scores (0.34, se = 0.09) that were made by weighing and averaging all scores (0 to 4) given to 9 clinical symptoms of IBH on 7 locations of a horse. Grandinson *et al.* (2006) estimated a heritability of 0.26 based on 4 categories; classifying IBH into 3 or 2 categories resulted in decreased heritabilities (0.12 to 0.15). However, it is unknown whether these heritabilities indeed differ, because Grandinson *et al.* (2006) did not report standard errors. The results suggest that scoring IBH as the absence or presence of clinical symptoms is sufficient for genetic analysis of IBH. However, more accurate heritability estimates might be expected when better methods of scoring IBH become available to detect affected or even sensitized horses on a continuous scale independent of the presence of *Culicoides* spp.

3.3.3 Repeatability

The estimated repeatability was 0.30 (Table 3.2) and indicates an upper limit to the heritability of IBH in the Dutch Shetland pony population, because repeatability

Table 3.2 Additive genetic variance (σ_a^2), permanent environmental variance (σ_{pe}^2), error variance (σ_e^2), and phenotypic variance (σ_p^2) along with heritability on the observed binary scale (h_{obs}^2), heritability on the underlying continuous scale (h_{und}^2), and repeatability (r) of insect bite hypersensitivity.

	Estimate[a]	
Parameter	Model 1	Model 2
Variance		
σ_a^2	0.006	0.004
σ_{pe}^2	0.017	0.017
σ_e^2	0.055	0.055
σ_p^2 (se)	0.078 (0.001)	0.076 (0.001)
Heritability		
h_{obs}^2 (*se*)	0.08 (0.02)	0.05 (0.02)
h_{und}^2 (se_{und})[b]	0.24 (0.06)	0.17 (0.05)
Repeatability		
r (*se*)	0.30 (0.02)	0.28 (0.02)

[a]Model 1 = the model excluding a fixed class effect of the inspector; model 2 = the model including a fixed class effect of the inspector.

[b] $se_{und} = se_{obs} \frac{[p(1-p)]}{z^2}$; se after transformation according to Dempster and Lerner (1950).

expresses the proportion of variance that is due to permanent environmental and genetic differences among individuals (Falconer and Mackay, 1996). Repeatability also indicates that including repeated observations of IBH clinical symptoms will improve the accuracy of breeding values for IBH.

The estimated repeatability was lower than expected; only 27.2% of the mares with IBH clinical symptoms during the first observation also showed clinical symptoms during the second observation (Table 3.3). This percentage was expected to be greater, because sensitization to an allergen develops at a young age (Holgate and Polosa, 2008), and once affected, IBH is considered to be permanent (Anderson *et al.*, 1991). Clinical symptoms of IBH were present during the first observation and absent during the second observation, possibly because of yearly and monthly fluctuations in environmental factors related to the presence and activity of *Culicoides* spp. (Anderson *et al.*, 1993). Within regions, locally large differences in IBH prevalence may arise, in part because *Culicoides* spp. are weak fliers (Braverman, 1988; Gortel, 1998), and, therefore, stay within a certain distance of the place where they were born. Unfortunately, month of scoring during the first and second observation was only known for 16 mares, because the date of scoring was not registered in 2003. To be certain that mares have been exposed to *Culicoides* spp., and, therefore, show clinical symptoms before they are scored for IBH, the Dutch Shetland Pony Studbook is advised not to score mares early in the *Culicoides* spp. season. Clinical symptoms of IBH were present during the first observation and absent during the second observation, in part because of the changing of environments caused by the sale of mares (9.5%) with repeated observations. None of the mares that showed clinical symptoms during the first observation and were moved subsequently showed clinical symptoms during the second observation (data not shown). These mares were probably sensitized, but were moved to an environment where *Culicoides* spp. are only occasionally present or even absent (van Grevenhof *et al.*, 2007), and, thus, did not show clinical symptoms during the second observation. Intervention by owners, such as stabling mares or using eczema blankets to avoid contact with *Culicoides* spp., is of minor importance because most Shetland mares (97.9%) are kept on pasture (Table 3.1), and eczema blankets for Shetland ponies have only recently become available.

3.3.4 IBH scoring method

Scoring of mares by inspectors based on clinical symptoms may not be the most accurate method, but scoring of clinical symptoms is still the gold standard for detecting IBH (Baselgia *et al.*, 2006), and allows inspectors to score a large number

Table 3.3 Comparison of the absence or presence of insect bite hypersensitivity clinical symptoms during the first and second observations for mares with repeated observations in percentage and number of mares (in parentheses).

	Clinical symptoms: second observation		
Clinical symptoms: first observation	Absent	Present	Total
Absent	94.8 (1,374)	5.2 (76)	100 (1,450)
Present	72.8 (75)	27.2 (28)	100 (103)

of mares. Because inspectors were trained by a veterinarian to score clinical symptoms of IBH, it is reasonable to assume that positive scores indicate IBH. However, other causes of pruritus resulting in similar clinical symptoms cannot be ruled out entirely.

One of the limiting conditions was to develop an IBH scoring method that could be implemented in the routine inspection system of the Dutch Shetland Pony Studbook. Foal inspections seemed most appropriate with respect to preselection.

Although only foals are inspected during foal inspections at home, mares (dams) should be present, whereas during organized mare inspections, the mares themselves are inspected. Therefore, there is less preselection when mares are scored for IBH during foal inspections compared with mares being scored at mare inspections.

As a consequence of scoring IBH during foal inspections, geldings and stallions were not considered. Most of the breeding stallions are kept stabled, and, therefore, are less exposed to *Culicoides* spp. Attractiveness of stallions, geldings, and mares to *Culicoides* spp. could differ, but from the literature it appears that sex differences are not to be expected (e.g. Halldórdsóttir and Larsen, 1991; Björnsdóttir *et al.*, 2006). Braverman *et al.* (1983) and Lange (2004) found a significant effect of sex on IBH prevalence, but Braverman *et al.* (1983) concluded that stallions were more often affected, whereas Lange (2004) concluded that mares were more often affected. In contrast, many researchers (e.g. Anderson *et al.*, 1988; Halldórdsóttir and Larsen, 1991; Steinman *et al.*, 2003; Björnsdóttir *et al.*, 2006) did not find a significant effect of sex on IBH prevalence.

3.3.5 Selection effect

A selection effect concerning IBH was found in the data and illustrates the possibility of selection against IBH. The IBH prevalence in mares with a single observation (10.3%) was significantly greater than the IBH prevalence in mares with

2 (7.3%) and 3 observations (5.1%). The number of observations per mare indicates the number of offspring a mare had during the time span of the study, because mares were scored during foal inspections. Mares with 2 or 3 observations are likely to be used frequently for breeding. Therefore, Dutch Shetland breeding mares more frequently used for breeding have decreased IBH prevalences.
As a result of this selection effect, the data set probably contained fewer affected Shetland ponies compared with the number affected in the entire population. This could have resulted in a slight underestimation of the heritability and repeatability of IBH in the Dutch Shetland pony population.

3.3.6 Conclusions and implications

Based on clinical symptoms, IBH is a heritable trait in the Dutch Shetland pony population. Therefore, the IBH prevalence in this population can be decreased by selection. The estimated repeatability indicates that including repeated observations of the clinical symptoms of IBH will improve the accuracy of breeding values for IBH.
The IBH scoring method used could be implemented in the routine inspection system of a studbook. To be certain that horses have been exposed to *Culicoides* spp., and therefore show clinical symptoms, it is advised not to score horses early in the *Culicoides* spp. season. However, more accurate or even greater heritability estimates might be expected when better methods other than subjective scoring of IBH become available to detect affected or even sensitized horses on a continuous scale independent of the presence of *Culicoides* spp. It seems useful, therefore, to develop methods for early and accurate diagnosis of IBH and to identify genomic regions involved in IBH sensitivity to efficiently select against horses susceptible for IBH. Selection against IBH will decrease economic losses for breeders due to disfiguration of their horses and possible veterinary costs, and, moreover, will increase animal welfare.

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4

Genetic parameters of insect bite hypersensitivity in Dutch Friesian broodmares

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Abstract

Insect bite hypersensitivity (IBH) is a seasonal allergic skin disease in horses caused by bites of certain *Culicoides* spp. The aim of our study was to investigate the maternal effect on IBH and to estimate the heritability and repeatability of IBH in the Dutch Friesian horse population. Data consisted of 3,453 Dutch Friesian broodmares with 3,763 visual observations on IBH clinical symptoms scored by 12 inspectors during organized foal inspections in 2004 and 2008. Nine percent of the mares (n = 310) were scored in both years. Mares descended from 144 sires and 2,554 dams and 26.2% of the dams (n = 669) had more than 1 offspring in the data set (range: 2-6). Insect bite hypersensitivity was analyzed as a binary trait with a threshold animal model with and without a maternal effect, using a Bayesian approach. Observed IBH prevalence in Dutch Friesian broodmare population was 18.2%. Heritability on the liability scale was 0.16 (sd = 0.06); heritability on the observed scale was 0.07; and repeatability was 0.89 (sd = 0.03). Maternal effect was 0.17 (sd = 0.06) and significantly differed from zero, although the animal model without a maternal effect fitted the data better. These results show that genetic and permanent environmental factors affect IBH in Dutch Friesian horses. The dam affected the IBH development of her offspring through an additive genetic influence but also by being part of their rearing environment.

Key words: *Culicoides* species, heritability, horse, insect bite hypersensitivity, maternal effect, summer eczema

4.1 Introduction

Insect bite hypersensitivity (IBH) is a common allergic skin disease in horses caused by bites of certain *Culicoides* spp. Affected horses develop an intense pruritus, which results in self-inflicted trauma. Common clinical symptoms are therefore hair loss, excoriation, scaling, thickening of skin, and open wounds (Björnsdóttir *et al.*, 2006; van den Boom *et al.*, 2008). Welfare of affected horses is reduced and some horses are unsuitable for riding and showing. Severely affected horses are occasionally euthanized (Anderson *et al.*, 1988; Gortel, 1998).

The etiology of IBH is multifactorial in origin. The heritability of IBH on the observed binary scale was 0.08 in Icelandic horse populations and Dutch Shetland ponies (Table 4.1). Offspring from the same dam are subject to a common maternal environment during the first stages of their life and therefore often have similar phenotypes as a result of shared genes and maternal environment (Falconer and Mackay, 1996; Kruuk and Hadfield, 2007). Results from Unkel *et al.* (1987) suggest that a maternal effect might contribute to the development of IBH.

Susceptibility to allergies in humans is presumably highly influenced by events occurring early in life (Holt and Jones, 2000; Halken, 2003). For instance, early life and long-term exposure to farming environments has a strong protective effect against the development of allergy in humans (Holgate and Polosa, 2008). Rearing conditions during the first stages of the life of a horse are expected to affect the development of IBH later in life. The aim of our study was therefore to investigate the effect of rearing conditions during early life on IBH including the maternal effect (as part of rearing conditions) and to estimate the heritability and repeatability of IBH in the Dutch Friesian horse population.

4.2 Materials and methods

Collection of IBH scores was a noninvasive method performed during routine foal inspections. Animal Care and Use Committee approval was therefore not required.

4.2.1 Population

Dutch Friesian broodmares were visually scored for IBH clinical symptoms by inspectors during organized foal inspections in 2004 and 2008. Data were a representative sample of the Dutch Friesian mare population because mares originated from 87 out of 90 regions (96.7%) across the Netherlands, where regions were defined based on 2-digit postal codes, and all mares with a foal were scored for IBH. Data were therefore an unselected sample of the population. Data from 3 mares with a missing value for age, month of scoring, or inspector were deleted.

Table 4.1 Overview of genetic analyses of insect bite hypersensitivity (IBH) in different horse populations.

Population	Parents	Method[a]	IBH observation	Prevalence	Trait[b]	h^2_{obs} [c]	h^2_{liab} [c]
Unkel et al. (1987)							
984 Icelandic horses born in Germany	36 sires 362 dams	LSM using PHS data	Owner	18.1%	0-1 scale	0.08 (0.07) 0.12 (0.08)	
Eriksson et al. (2008)							
1,250 Icelandic horses born in Sweden	33 sires 942 dams	TSM	Owner	8.0%[d]	0-1 0-1-2 0-1-2-3	0.08 0.09 0.10	0.27 (0.17) 0.30 (0.19) 0.33 (0.19)
Schurink et al. (2009)							
6,073 Dutch Shetland ponies	984 sires 4,455 dams	LAM	16 inspectors trained by a veterinarian	8.8%	0-1 0-1-2	0.08 (0.02) 0.08	0.24 (0.06)

[a]LSM = linear sire model; PHS = paternal half-sib; TSM = threshold sire model; LAM = linear animal model.

[b]0, 1: unaffected to affected; scale: judged according to the number of affected parts of the body, ranging from mild to severe where a horse is mildly affected when only tail or mane or tail and mane are affected, and severely affected when the entire body contains large affected areas; 0, 1, 2 (Eriksson *et al.*, 2008): unaffected, mild IBH, moderate to severe IBH, respectively; 0, 1, 2, 3: unaffected, mild IBH, moderate IBH, severe IBH, respectively; 0, 1, 2 (Schurink *et al.*, 2009): unaffected, dubious or mild symptoms, clear symptoms, respectively.

[c] h^2_{obs} = heritability on the observed scale; h^2_{liab} = heritability on the liability scale; in parentheses se (linear model) or sd (threshold model), depending on analysis method used.

[d]Mild IBH: 4.7%; moderate IBH: 2.6%; severe IBH: 0.7%.

Data from 77 mares from mainly coastal regions without IBH (16 out of 87) were deleted because *Culicoides* spp. were probably absent within these regions (van Grevenhof *et al.*, 2007), which enabled estimation of model effects. Number of observations per region varied from 3 to 700. Seventeen out of 71 regions (23.9%) contained less than 10 observations and 9 out of 71 regions (12.7%) contained over 100 observations. After editing, data consisted of 3,763 IBH scores on 3,453 Dutch Friesian broodmares (Table 4.2); 310 mares (9.0%) were scored in both years.
Average age was 9.0 yr (sd = 4.2 yr) and varied from 3 to 25 yr (Table 4.2). Data from 3- and 4-yr-old mares were grouped together (≤ 4 yr) as well as data from 20 yr and older mares (≥ 20 yr) because of few observations. Most mares were scored in July, August, and September; some mares were scored in June and October (Table 4.2).
Mares descended from 144 sires and 2,554 dams. Paternal half-sib groups ranged from 1 to 149 individuals. In total 669 dams (26.2%) had more than 1 offspring in the data set (range: 2-6). Pedigree was for 97.9% complete and used up to the 4th ancestral generation. Inbreeding coefficients were calculated using full pedigree data, but did not significantly affect IBH.

4.2.2 Trait definition of IBH

Friesian broodmares were visually scored by 8 inspectors in 2004 and 4 inspectors in 2008. Number of mares scored per inspector varied from 13 (0.5%) to 1,361 (49.5%) in 2004 and from 49 (4.8%) to 425 (42.0%) in 2008. Scoring of IBH clinical symptoms was made as uniform as possible with help from a veterinarian during a training session in 2004. Inspectors visually scored the clinical symptoms (e.g. hair loss, thickened skin, bald spots, crusting, scaling, and excoriation) on IBH-sensitive locations such as the crest and base of the tail. Insect bite hypersensitivity status of mares was scored in 3 categories: 0 = absence of clinical symptoms, 1 = dubious or mild clinical symptoms, and 2 = clear clinical symptoms. Categories 1 and 2 were grouped together because percentage of mares with score 1 was limited (5.1%); resulting IBH scores were analyzed as a binary trait.

4.2.3 Statistical analysis

Model

Data were analyzed with a threshold animal model. A threshold model assumes the presence of an underlying nonobservable continuous variable, known as liability. When liability is equal to or below a certain threshold, an animal will be unaffected;

Table 4.2 Data description.

	Year of scoring		
Variable	2004	2008	Total
Observations,[a] n (%)	2,751 (73.1)	1,012 (26.9)	3,763
Observed IBH[b] prevalence, %	18.5	17.2	18.2
Mean age, yr (range)	8.8 (3-25)	9.3 (4-24)	9.0 (3-25)
Month of scoring, n (%)			
June	167 (6.1)	40 (4.0)	207 (5.5)
July	806 (29.3)	244 (24.1)	1,050 (27.9)
August	957 (34.8)	404 (39.9)	1,361 (36.2)
September	661 (24.0)	267 (26.4)	928 (24.6)
October	160 (5.8)	57 (5.6)	217 (5.8)

[a]Percentage of total number of observations.
[b]IBH = insect bite hypersensitivity.

when the liability is above the threshold, an animal will be affected. In formula, this is

$$y_i = \begin{cases} 0 & \text{if } \lambda_i \leq \tau \\ 1 & \text{if } \lambda_i > \tau \end{cases},$$

where $y_i = 0$ denotes an unaffected mare and $y_i = 1$ denotes an affected mare, depending on whether their liability (λ_i) exceeded the fixed threshold (τ) for the manifestation of IBH.

Fixed effects

Because data were binary, a binomial distribution with an inverse probit link function was used. The linear predictor (η_{ijkl}) was:

$$\eta_{ijkl} = \mu + age_i + region_j + yearmonth_k + inspector_l,$$

where μ = population mean on liability scale; age_i = fixed effect of the age of a Friesian broodmare in years (i = ≤4, 5, 6, …, ≥20); $region_j$ = fixed effect of region (j = 1, 2, 3, …, 71); $yearmonth_k$ = fixed combined effect of year and month of scoring (k = 1, 2, 3, …, 10); $inspector_l$ = fixed effect of inspector (l = 1, 2, 3, …, 12). Fixed model effects were estimated using GLIMMIX procedure that fitted generalized linear mixed models (SAS Inst. Inc., Cary, NC).

4.2.4 Estimation of variance components

For the threshold models the underlying liabilities were sampled using Markov Chain Monte Carlo methodology through a Gibbs sampling algorithm. The threshold and residual variance were set to respectively $\tau = 0$ and $\sigma_e^2 = 1$. Uninformative (flat) priors were assumed for all random model effects, being inverse χ^2 distribution. Shrinkage of fixed model effects was used to prevent poor convergence (Janss, 2008). Marginal posterior distributions of heritability, repeatability, and maternal effect were assessed using iBay software (Janss, 2008). An inverse probit link function, $\mu_i = \Phi(\eta_i)$, was used to relate the linear predictor (η_i), the IBH liability, to the conditional mean on the observed scale (μ_i), where Φ is the cumulative distribution function of the standard normal distribution and μ_i is the expected response on the observed scale (Kachman, 2000). The linear predictor (η_{ijklmn}) of the animal model was:

η_{ijklmn} = μ + age_i + $region_j$ + $yearmonth_k$ + $inspector_l$ + pe_m + $animal_m$ + $maternal_n$,

where fixed effects are as described previously; pe_m = the random permanent environment of the mth animal (m = 3,453) with $\sim N(0, \mathbf{I_{pe}}\sigma_{pe}^2)$; $animal_m$ = the random additive genetic effect of the mth animal (m = 3,453) with $\sim N(0, \mathbf{A}\sigma_a^2)$; and $maternal_n$ = the random maternal effect of the nth dam (n = 2,554) with $\sim N(0, \mathbf{I_m}\sigma_m^2)$; where $\mathbf{I_{pe}}$ and $\mathbf{I_m}$ are identity matrices of the appropriate dimensions; $\mathbf{A}$ is a matrix of additive genetic relationships among Friesian horses in our data; σ_{pe}^2 = permanent environmental variance, σ_a^2 = additive genetic variance, and σ_m^2 = maternal variance. Residual variance of liabilities (σ_e^2) was fixed to 1, related to the inverse probit link function. Phenotypic variance for the animal model with maternal effect was $\sigma_p^2 = \sigma_{pe}^2 + \sigma_m^2 + \sigma_a^2 + \sigma_e^2$. Random effects were considered to be independent (no covariance). An animal model without the random maternal effect was run as well. Phenotypic variance for the animal model without maternal effect was $\sigma_p^2 = \sigma_{pe}^2 + \sigma_a^2 + \sigma_e^2$.

In total 10 independent chains of 210,000 cycles per chain were run, with a burn-in period of 10,000 cycles. Every 500th sample was stored giving a total of 4,000 samples. Saving every 100th sample hardly affected the results. Analysis of within and between chain variance for variance components estimates indicated an

effective number of samples of at least 347. Natural logarithm of Bayes Factor was used to compare models (Janss, 2008). Bayes Factor is penalized for the number of parameters in the model. The model with greater Bayes Factor is therefore better (Kass and Raftery, 1995; Janss, 2008).

4.2.5 Calculations of parameters derived from variance components

The correlation between repeated observations within a Friesian broodmare, known as repeatability (r), was calculated as

$$r = \frac{\sigma_a^2 + \sigma_{pe}^2}{\sigma_p^2}$$

where all variance components are as described previously. The numerator included σ_m^2 for the animal model with maternal effect.

Heritability on the liability scale (h_{liab}^2) was calculated as

$$h_{liab}^2 = \frac{\sigma_a^2}{\sigma_p^2}$$

where all variance components are as explained previously. To enable comparison with results from previous studies on the genetic background of IBH, heritability on the liability scale was transformed to heritability on the observed scale (h_{obs}^2) according to Dempster and Lerner (1950):

$$h_{obs}^2 = \frac{z^2 h_{liab}^2}{p(1-p)}$$

where z = the ordinate of a standard normal distribution at the threshold corresponding to the fraction of affected mares, which is p = 0.182.

Maternal effect was calculated as

$$\text{maternal effect} = \frac{\sigma_m^2}{\sigma_p^2}$$

where all variance components are as explained previously.

Calculations of parameters derived from estimated variance components was needed to obtain features of marginal posterior distributions (mean, sd, 95% highest posterior density region) of repeatability, heritability, and maternal effect by computing from each Gibbs sample the corresponding value of repeatability, heritability, or maternal effect and subsequently using these values when the posterior distributions were summarized.

4.3 Results and discussion

4.3.1 Observed IBH prevalence

Observed IBH prevalence was 18.5% in 2004 and 17.2% in 2008, on average 18.2% (Table 4.2). Prevalence in Friesian broodmares descending from affected dams or maternal granddams was greater (respectively 21.3 and 17.6%) than prevalence of mares descending from unaffected dams or maternal granddams (respectively 17.9 and 12.2%). An increased prevalence in horses descending from affected dams was also found in Icelandic horses (Eriksson *et al.*, 2008) and Shetland broodmares (Schurink *et al.*, 2009). Prevalence in paternal half-sib groups with more than 20 members varied from 0 to 34.9%. Prevalence in paternal half-sib groups in an Icelandic horse population varied from 0 to 30% (Eriksson *et al.*, 2008) and in a Shetland broodmare population from 0 to 37% (Schurink *et al.*, 2009).

4.3.2 Associated effects

Combined effect of year and month of scoring ($P = 0.003$), inspector ($P < 0.0001$), and region within the Netherlands ($P = 0.043$) had a significant effect on IBH prevalence. Age of the mare had a suggestive effect ($P = 0.078$).

Year and month of scoring

Predicted prevalence increased with month of scoring in 2004 from 14.0% in June to 23.7% in October. Predicted prevalence of month of scoring in 2008 fluctuated: 7.8% in June, 7.3% in July, 16.4% in August, 18.1% in September, and 3.2% in October. Distinct annual trends in IBH prevalence might be related to differences in presence and activity of *Culicoides* spp. between years and months, which could be caused by differences in environmental conditions (Anderson *et al.*, 1993; Takken *et al.*, 2008).

Inspector

Predicted prevalence of inspector varied from 11.6 to 35.0%. Within a region, 1 to 10 inspectors scored the Friesian broodmares. In 3 out of 71 regions only 1

inspector per region scored the mares (n = 15). In 16 regions, 7 to 10 inspectors per region scored 2,786 mares (74.0% of all observations). Inspector and region within the Netherlands were therefore not confounded. Thus, differences in IBH prevalence between inspectors were not explained by regional differences in IBH prevalence. Scoring of IBH clinical symptoms was uniformed with help from a veterinarian during a training session in 2004, but only a portion of the inspectors was able to join. Because IBH scoring was a subjective method, inspector was included in the analysis.

Region

Predicted prevalence in regions with more than 20 observations varied from 1.2 to 28.6%. Most low-risk regions were found in coastal areas of the Netherlands, whereas most high-risk regions were found in the provinces Gelderland, Noord-Brabant, Overijssel, and Limburg. This finding agrees with results from van Grevenhof *et al.* (2007) and van den Boom *et al.* (2008) for various horse breeds (e.g. Friesian horses, Icelandic horses, Haflinger horses, Warmblood horses, Shetland ponies). Regional differences in IBH prevalence were observed by other researchers (e.g. Broström *et al.*, 1987; van Grevenhof *et al.*, 2007; Eriksson *et al.*, 2008; van den Boom *et al.*, 2008). van Grevenhof *et al.* (2007) found that habitat

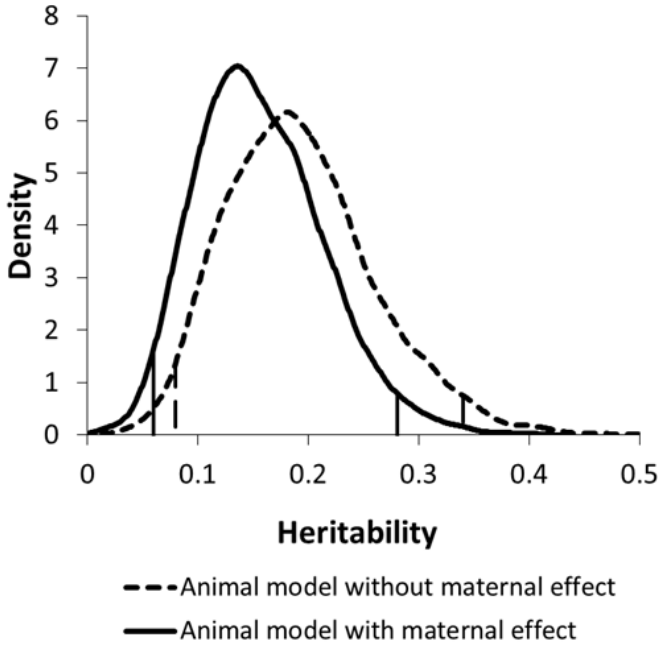

Figure 4.1 Estimated marginal posterior distribution of heritability on the liability scale (95% highest posterior density regions between vertical lines) estimated with an animal model with (solid line) and without a maternal effect (dashed line).

and climate factors, which included information about vegetation, soil type, rainfall, number of warm and cold days within regions of the Netherlands, significantly affected IBH prevalence in a region. Environmental conditions within regions affect the presence and activity of *Culicoides* spp. and thereby the observed IBH prevalence.

Age

Predicted prevalence in Friesian broodmares up to 4 yr was 27.9%. Predicted prevalence increased to 38.8% in 8-yr-old mares and subsequently decreased to 18.1% in 20 yr and older mares. Although often an increase in prevalence with age was found (e.g. Halldórsdóttir and Larsen, 1991; Steinman *et al.*, 2003), our results might reflect an effect of selection, where affected Friesian broodmares are culled from breeding.

4.3.3 Repeatability, heritability and maternal effect

Estimates of variance components, repeatability, heritability, and maternal effect for the animal model with and without maternal effect are shown in Table 4.3. Estimated marginal posterior distributions of heritability on the liability scale and maternal effect are shown in Figures 4.1 and 4.2. Estimates of repeatability, heritability, and maternal effect will be described and discussed separately in the subsequent sections.

Repeatability

Investigation of uncorrected repeated observations on 310 Friesian broodmares (Table 4.4) showed that 78.7% of the mares (n = 244) had a similar IBH observation in 2004 and 2008. However, 14.9% (n = 40) of mares without clinical symptoms in 2004 had developed clinical symptoms in 2008. Also, 61.9% (n = 26) of mares with clinical symptoms in 2004 did not have clinical symptoms in 2008. Schurink *et al.* (2009) analyzed uncorrected phenotypes of Dutch Shetland broodmares and found that 5.2% (n = 76) of mares without symptoms in the first year had developed symptoms in the second year and 72.8% (n = 75) of mares with symptoms in the first year did not have symptoms in the second year. When exposed to *Culicoides* spp., clinical symptoms rarely disappear with age (Anderson *et al.*, 1988). Owners possibly started applying preventive measures after a mare showed IBH, which resulted in the reduction of clinical symptoms. Information on application of preventive measures by owners was not available to confirm the impact. Year and month of scoring, region, inspector, and age of the mares were known and

Table 4.3 Direct additive genetic variance (σ_a^2), permanent environmental variance (σ_{pe}^2), maternal variance (σ_m^2), phenotypic variance (σ_p^2); posterior mean, posterior standard deviation (sd), and 95% highest posterior density region (HPDR) of heritability on the liability scale (h_{liab}^2), repeatability (r), and maternal effect of insect bite hypersensitivity (IBH); heritability on the observed scale (h_{obs}^2); estimates are shown for an animal model without and with a maternal effect.

	Variance components[a]				h_{liab}^2		h_{obs}^2 [b]	r [c]		Maternal effect	
Model	σ_a^2	σ_{pe}^2	σ_m^2	σ_p^2	Mean (sd)	95% HPDR		Mean (sd)	95% HPDR	Mean (sd)	95% HPDR
Animal model without maternal effect	1.78	6.43	-	9.21	0.19 (0.07)	0.08-0.34	0.09	0.88 (0.03)	0.82-0.93	-	-
Animal model with maternal effect	1.51	5.43	1.71	9.65	0.16 (0.06)	0.06-0.28	0.07	0.89 (0.03)	0.83-0.93	0.17 (0.06)	0.05-0.30

[a]Animal model without maternal effect: $\sigma_p^2 = \sigma_{pe}^2 + \sigma_a^2 + \sigma_e^2$; animal model with maternal effect: $\sigma_p^2 = \sigma_{pe}^2 + \sigma_m^2 + \sigma_a^2 + \sigma_e^2$; where $\sigma_e^2 = 1$, related to the inverse probit link function.

[b]Heritability on the observed scale after transformation according to Dempster and Lerner (1950); $h_{obs}^2 = \frac{z^2 h_{liab}^2}{p(1-p)}$.

[c]Repeatability calculated using animal model without maternal effect: $r = \frac{\sigma_a^2 + \sigma_{pe}^2}{\sigma_p^2}$, and using animal model with maternal effect: $r = \frac{\sigma_a^2 + \sigma_{pe}^2 + \sigma_m^2}{\sigma_p^2}$.

Table 4.4 Comparison of the absence or presence of observed insect bite hypersensitivity (IBH) clinical symptoms in 2004 and 2008 for Friesian broodmares with repeated observations in number of mares and percentage (in parentheses).

	IBH clinical symptoms 2008		
IBH clinical symptoms 2004	Absent	Present	Total
Absent	228 (85.1)	40 (14.9)	268 (100)
Present	26 (61.9)	16 (38.1)	42 (100)

contributed to changes in IBH status of Friesian broodmares between years. The model for estimation of repeatability (and all other parameters) corrected for these effects.

Mean repeatability estimate in Friesian broodmares was 0.89 in the animal model with a maternal effect and 0.88 in the animal model without a maternal effect (Table 4.3). Insect bite hypersensitivity is considered to be a permanent condition (Anderson *et al.*, 1991). A high repeatability was therefore expected. Repeatability in Dutch Shetland broodmares was 0.30 (se = 0.02; Schurink *et al.*, 2009). However, repeatability estimate of Schurink *et al.* (2009) is likely underestimated due to limited possibilities to correct for fixed effects and confounding of genetic effects, region and inspector.

Heritability

Mean heritability estimate on the liability scale was 0.16 in the animal model with a maternal effect and 0.19 in the animal model without a maternal effect (Table 4.3). Mean heritability estimate on the liability scale estimated with a sire and sire-dam model were respectively 0.17 (sd = 0.08) and 0.21 (sd = 0.12; data not shown). Heritability significantly differed from 0, because 95% highest posterior density regions excluded 0. Mean heritability on the observed scale was 0.07 in the animal model with a maternal effect and 0.09 in the animal model without a maternal effect (Table 4.3). Heritability on the liability scale is less compared with estimates from Eriksson *et al.* (2008) and Schurink *et al.* (2009; Table 4.1). Estimates of heritability on the observed scale depend on prevalence within a population, which was 18.2% in Friesian broodmares, 18.1% in German-born Icelandic horses (Unkel *et al.*, 1987), 8.0% in Swedish-born Icelandic horses (Eriksson *et al.*, 2008), and 8.8% in Shetland mares (Schurink *et al.*, 2009; Table 4.1). Heritability on the observed scale in Friesian broodmares equals estimates from Unkel *et al.* (1987), Eriksson *et al.* (2008), and Schurink *et al.* (2009), despite differences in recording of phenotype, prevalence, analysis method used, and investigated breed and population (Table 4.1).

Maternal effect

Mean maternal effect significantly differed from 0 and composed 17% of the variance (Table 4.3), which is comparable in size with the variation explained by genetic factors (i.e., heritability). Mean maternal effect estimated with a sire and sire-dam model composed, respectively, 17 and 21% of the variance (data not shown). Besides a direct additive genetic influence, the dam also affected the IBH development of her offspring by being part of their rearing environment. Unkel *et al.* (1987) suggested a similar effect of a common maternal effect on IBH (0.20) in Icelandic horses because ANOVA using full-sib data indicated that IBH heritability based on dam variance was greater (h_d^2 = 1.04) compared with heritability based on sire variance (h_s^2 = 0.23). The common maternal effect estimated by Unkel *et al.* (1987) included both maternal genetic and maternal environmental effects.

Possible causes of a maternal effect might be related to maternal features like transfer of various immunological substances via colostrum, to environmental aspects experienced during early life such as habitat related to allergen exposure, to management by breeders such as feeding or stabling, and to genetic ability. Susceptibility to allergies is presumably highly influenced by events occurring early in life (Holt and Jones, 2000; Halken, 2003). Maternal equine IgE antibodies against *Culicoides* allergens are passively transferred from IBH-affected mares to their foals via colostrum. However, consequences of transferred maternal equine IgE are unknown (Wagner *et al.*, 2006; Marti *et al.*, 2009).

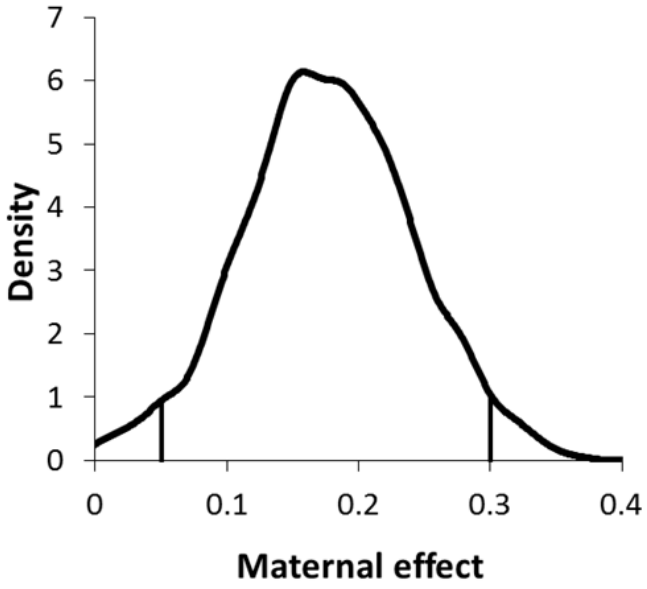

Figure 4.2 Estimated marginal posterior distribution of maternal effect (95% highest posterior density region between vertical lines).

Environmental conditions experienced by maternal (half)-sibs during early life, such as allergen exposure related to habitat, or management by breeders, such as feeding or stabling, might result in maternal (half)-sibs with similar phenotypes caused by shared genes, but also due to shared environment. Region of the dam was known for 30.0% (n = 1,130) of the Friesian broodmares. In total, 57.7% (652 out of 1,130) of the broodmares were kept within the same region as their dam. Maternal effect might therefore include a regional effect because some progeny remain within the same region of their dam, although fixed effect of region within the Netherlands remained significant. Maternal effect might also represent a farm effect, but estimates of maternal effect differed between dams within a farm.

4.3.4 Trait definition of IBH

Friesian broodmares with dubious or mild clinical symptoms (category 1) and clear clinical symptoms (category 2) were considered to be affected and IBH scores were analyzed as a binary trait. When only Friesian broodmares with clear clinical symptoms (category 2) were considered to be affected, heritability, repeatability, and maternal effect were in the range of estimates presented in Table 4.3 (data not shown). Heritability in Dutch Shetland broodmares was equal when IBH was analyzed as a binary trait or on a 0, 1, 2 scale (Schurink *et al.*, 2009). Eriksson *et al.* (2008) classified severity of IBH symptoms in Icelandic horses into several categories (mild-severe) and found that heritability was greater (h^2_{liab} = 0.33) compared with heritability of IBH analyzed as a binary trait (h^2_{liab} = 0.27). However, sd were large (Table 4.1), and all 95% highest posterior density regions overlapped (Eriksson *et al.*, 2008). The benefit of scoring severity of IBH symptoms for genetic research is limited because only phenotypes of affected horses, the minority of a population, are subdivided. The majority of horses in a population are unaffected (often >80%; i.e., without clinical symptoms) and therefore have a similar phenotype, although sensitivity to IBH in unaffected horses can differ greatly. Research on IBH will therefore benefit from a reliable diagnostic or DNA test that can distinguish horses according to their sensitivity to IBH, independent of observed clinical symptoms.

4.3.5 Model comparison

The animal model with and without maternal effect obtained similar heritability and repeatability estimates (Table 4.3); 95% highest posterior density region of heritability and repeatability overlapped. Clément *et al.* (2001) simulated data to investigate the effect of model adequacy on the estimation of genetic parameter

for traits affected by maternal effects and found that heritability was overestimated when a maternal (genetic or genetic and environmental) effect existed but was neglected. Similar results were obtained by Kruuk and Hadfield (2007) in a simulated ungulate population. True direct additive genetic variance was 0.3, and true maternal environmental variance was 0.2. When maternal environmental effect was neglected, direct additive genetic variance was overestimated (0.52). Our results (Table 4.3) show that IBH heritability estimated with an animal model without maternal effect was only slightly greater (0.19) compared with heritability estimated with an animal model with maternal effect (0.16).

Heritability, repeatability, and maternal effect significantly differed from 0; all 95% highest posterior density regions excluded 0 (Table 4.3; Figures 4.1 and 4.2). However, natural logarithm of Bayes Factor was greater for the animal model without a maternal effect. The animal model without a maternal effect therefore fitted the data better, though data including more dams with at least 2 offspring will be required to demonstrate a significant improvement of the model when including a maternal effect and to separate maternal genetic and maternal environmental effects.

4.3.6 Conclusions and implications

Insect bite hypersensitivity is a heritable trait in the Dutch Friesian horse population. Repeatability of IBH scores is high (0.89). Maternal effect on IBH (0.17) is comparable in size with the heritability estimate (0.16). Besides an additive genetic influence, the dam also affected the IBH development of her offspring by being part of their rearing environment.

Although selection against IBH is possible, a traditional breeding program based only on scoring clinical symptoms is inefficient. Progeny testing is needed to reliably estimate the genetic potential of stallions. However, progeny testing will seriously increase generation interval because offspring have to be old enough (at least 3 yr of age) and exposed to *Culicoides* spp. to be able to show IBH clinical symptoms. Genomic information can determine the genetic potential of an individual more reliably, at a younger age, and independent of the presence of *Culicoides* spp. Including genomic information in a breeding program against IBH will improve response to selection and thereby decrease the number of affected horses more rapidly compared with a traditional breeding program based on progeny testing.

4.4 Acknowledgements

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Genome-wide association study of insect bite hypersensitivity in Dutch Shetland pony mares

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Abstract

Insect bite hypersensitivity (IBH) is the most common allergic disease present in horses worldwide. It has been shown that IBH is under genetic control, but the knowledge of associated genes is limited. We conducted a genome-wide association study to identify and quantify genomic regions contributing to IBH in the Dutch Shetland pony population. A total of 97 cases and 91 controls were selected and matched on withers height, coat colour and pedigree to minimise the population stratification. A blood sample was collected from participating Shetland pony mares, their IBH phenotype was scored and the owner filled in a questionnaire. A total of 40,021 single-nucleotide polymorphisms (SNPs) were fitted in a univariable logistic model fitting an additive effect. Analysis revealed no effects of population stratification. Significant associations with IBH were detected for 24 SNPs on 12 chromosomes [$-\log_{10}(P) > 2.5$]. Odds ratios of allele substitution effects of the unfavourable allele were between 1.94 and 5.95. The most significant SNP was found on chromosome 27, with an odds ratio of 2.31 and with an allele frequency of the unfavourable allele of 0.72 in cases and 0.53 in controls. Genome-wide association studies on additional horse populations are desired to validate the identified associations, to identify the genes involved in IBH and to develop genomic tools to decrease IBH prevalence.

Key words: genome-wide association study, insect bite hypersensitivity, matched case-control design

5.1 Introduction

Insect bite hypersensitivity (IBH) is the most common allergic disease in horses and found in many countries throughout the world (Anderson *et al.*, 1988; Littlewood, 1998; Steinman *et al.*, 2003). Observed prevalence of IBH is, for instance, 8.1% in Swedish-born Icelandic horses (Eriksson *et al.*, 2008), 8.8% in Shetland pony mares (Schurink *et al.*, 2009) and 18.2% in Friesian broodmares (Schurink *et al.*, 2011). Sensitive horses develop a severe itch after exposure to bites of certain midges; *Culicoides* spp. are reported as the most common cause of IBH (Riek, 1954; Fadok and Greiner, 1990). *Culicoides* spp. are most active at sunset (van der Rijt *et al.*, 2008) and usually disappear during winter in countries with a temperate climate. The presence of *Culicoides* spp. is highly dependent on environmental conditions (Takken *et al.*, 2008).

The welfare of affected horses is seriously reduced. Common clinical symptoms in affected horses are hair loss, bald spots, excoriation, crusting, scaling and thickening of the skin (van den Boom *et al.*, 2008). Severe cases might suffer from open wounds and secondary infections. Use of affected horses, like showing and riding, can be restricted owing to discomfort and disfigurement (Gortel, 1998). Owners may encounter costs related to measures taken to prevent or limit the development of clinical symptoms, although fully effective measures are still unavailable (van den Boom *et al.*, 2008). The commercial value of affected horses is reduced (Broström *et al.*, 1987).

Scoring clinical symptoms is still the gold-standard for detecting IBH (Baselgia *et al.*, 2006). Analysing the severity of clinical symptoms hardly affects heritability estimates (Eriksson *et al.*, 2008; Schurink et al,. 2009) and therefore seems unnecessary, probably because only a small percentage of horses within a population are affected and preventive measures strongly influence the severity of clinical symptoms. Specific *in vitro* and *in vivo* tests were developed to detect IBH (e.g. Baselgia *et al.*, 2006; Wagner *et al.*, 2008; Sloet van Oldruitenborgh-Oosterbaan *et al.*, 2009), but test results often only support a clinical diagnosis. A case-control design, therefore, seems useful for treating the disorders like IBH that are difficult to phenotype. However, caution is needed when selecting cases and controls. Matching of cases with controls for certain features like ancestry is desired to avoid population stratification and thereby limit unwanted spurious associations in genome-wide association studies (e.g. Karlsson and Lindblad-Toh, 2008; McCarthy *et al.*, 2008).

A genetic component of IBH has been confirmed in various horse breeds (Unkel *et al.*, 1987; Eriksson *et al.*, 2008; Schurink *et al.*, 2009; Schurink *et al.*, 2011). Insect

bite hypersensitivity heritability estimates on the liability scale vary from 0.16 in Friesian horses (Schurink *et al.*, 2011) to 0.33 in Swedish-born Icelandic horses (Eriksson *et al.*, 2008). Knowledge of genes contributing to the genetic variation in IBH is limited. Recently, Andersson *et al.* (2012) conducted a candidate gene approach and identified an association between equine leukocyte antigen (ELA) class II region and IBH in Icelandic horses and Exmoor ponies. The same allele (*COR112:274*) was associated with IBH in both breeds, and homozygosity across the entire ELA class II region increased the risk of developing IBH. Recently, the horse's (*Equus caballus*) genome was sequenced (Wade *et al.*, 2009), which enabled the development of a genotyping chip with 50,000 single nucleotide polymorphisms (SNPs) (Illumina Inc.), thereby presenting us with the opportunity to conduct a genome-wide association study to increase our knowledge about genes involved in IBH. Knowledge of genomic associations with IBH could increase the efficiency of breeding for decreased IBH prevalence through genomic selection using only significant markers or all markers. Moreover, an increased knowledge of genes involved in IBH will contribute to a better understanding of the underlying biology and thereby to improved prevention, diagnosis and therapy of IBH. The aims of our study were to identify and quantify genomic associations with IBH in the Dutch Shetland pony population, using a genome-wide association approach.

5.2 Materials and methods

5.2.1 Study design

Shetland pony mares were selected according to a matched case-control design to minimise the effects of population stratification (the presence of multiple subgroups within the population that differ in both allele frequency and disease prevalence; for example, McCarthy *et al.* (2008)). Cases were defined as mares showing IBH clinical symptoms (mild to severe), whereas controls were free of symptoms. Cases and controls were matched on various factors (Table 5.1) to minimise potential population stratification. Mares were grouped by the Dutch Shetland Pony Studbook into four categories according to their withers height: mini (86 cm), small (87-92 cm), middle (93-98 cm) and tall (99-107cm).

The number of available cases and controls determines the power to detect a specific locus; power also depends on several (unknown) factors like inheritance, allele frequency and relative risk. We aimed at 150 cases and 150 controls to achieve reasonable power to detect genomic regions highly associated with IBH, but managed to collect data on 97 cases and 91 controls.

Table 5.1 Distribution [% (number)] of characteristics of Dutch Shetland pony mares over cases and controls investigated in 2009 and 2010.

Trait	Case	Control	Total
Number of mares	97	91	188
Year of scoring			
2009	79.4 (77)	79.1 (72)	79.3 (149)
2010	20.6 (20)	20.9 (19)	20.7 (39)
Month of scoring			
September	53.6 (52)	57.1 (52)	55.3 (104)
October	43.3 (42)	39.6 (36)	41.5 (78)
November	3.1 (3)	3.3 (3)	3.2 (6)
Veterinarian			
1	94.8 (92)	95.6 (87)	95.2 (179)
2	5.2 (5)	4.4 (4)	4.8 (9)
Pedigree			
Number of sires	80	81	125
Number of dams	94	88	176
Age, years			
Mean (sd)	7.0 (4.3)	8.5 (4.5)	7.7 (4.4)
Range	0 – 21	4 – 22	0 – 22
Withers height category			
Mini	26.8 (26)	25.3 (23)	26.0 (49)
Small	15.5 (15)	16.5 (15)	16.0 (30)
Middle	30.9 (30)	29.7 (27)	30.3 (57)
Tall	26.8 (26)	28.5 (26)	27.7 (52)
Coat colour			
Bay	3.1 (3)	5.5 (5)	4.3 (8)
Bay paint	1.0 (1)	2.2 (2)	1.6 (3)
Black	50.5 (49)	52.7 (48)	51.6 (97)
Black paint	6.2 (6)	4.4 (4)	5.3 (10)
Black roan	3.1 (3)	1.1 (1)	2.1 (4)
Blue dun	-	1.1 (1)	0.5 (1)
Chestnut	24.7 (24)	24.2 (22)	24.5 (46)
Chestnut paint	8.3 (8)	6.6 (6)	7.5 (14)
Chestnut roan	2.1 (2)	1.1 (1)	1.6 (3)
Golden dun	1.0 (1)	-	0.5 (1)
Palomino	-	1.1 (1)	0.5 (1)

5.2.2 Selection of cases and controls

In 2009, IBH phenotypes of Shetland pony mares were recorded by studbook inspectors during foal inspections. Owners of mares that scored positive (i.e. cases) were contacted later that year and asked to participate in our study. Participation consisted of filling in a questionnaire and allowing a veterinarian to collect blood from their affected mare(s). Enrolment of cases continued in 2010 when

Table 5.2 Number of case and control offspring per sire.

Number of case offspring per sire	Number of control offspring per sire	Number of sires
1	0	38
2	0	4
3	0	2
0	1	43
0	2	2
1	1	25
1	2	3
1	3	1
2	1	3
2	2	2
3	1	1
3	2	1

participants were recruited by the Dutch Shetland pony studbook through a publication in their magazine and on their website. This change of approach was needed because IBH phenotypes of Shetland pony mares were no longer recorded by studbook inspectors in 2010. The data collected from 2009 to 2010 contained 97 cases (Table 5.1).

Mares that had been exposed to *Culicoides* spp. but did not develop IBH clinical symptoms were used as controls. Controls were selected based on the following matching criteria: withers height, coat colour, proximity to a case and pedigree. Withers height clearly affects IBH in Shetland pony mares (Schurink *et al.*, 2009), although its reason remains unclear. Coat colours might differ in attractiveness to *Culicoides* spp., as Braverman *et al.* (1983) found that horses with dark coat colour were more often affected by IBH. Although age at onset of IBH varies greatly (Anderson *et al.*, 1988; Eriksson *et al.*, 2008; van den Boom *et al.*, 2008), average age at onset is considered to be between 2 and 4 years of age. Therefore, controls were required to be at least 4 years of age and at least 1-2 years at risk for developing IBH clinical symptoms. Proximity to a case was required, that is, on the same premises, to ensure exposure of controls to *Culicoides* spp., and thereby increases the reliability of the control phenotype, as sensitive individuals would have developed clinical symptoms in the presence of *Culicoides* spp. Controls were also genealogically matched by selecting paternal half-sibs of cases wherever possible to minimise potential population stratification (e.g. Hirschhorn and Daly, 2005; Karlsson and Lindblad-Toh, 2008). We collected blood from 91 controls (Table 5.1).

Data came from 99 mares (52.7% of the data) descending from 89 sires with only case(s) or control(s) among their offspring, and 89 half-sib mares (47.3% of the data) descending from 36 sires with both case(s) and control(s) among their offspring (Table 5.2). Cases and controls descended from a similar number of ancestors (Table 5.1) (average inbreeding coefficient of cases was 0.047 and of controls was 0.046).

Cases and controls were preferably present for 2 or more years on their current premises to ensure constant management practices (i.e. environment) and exposure to *Culicoides* spp. for a reasonable time. Most Shetland pony mares (93.1%) were present for 2 or more years on the current premises, 5.3% of the mares were present <2 years, and information on length of presence was lacking for 1.6% of the mares.

5.2.3 Participation

In total, 115 (4.1%) out of 2,809 mares scored IBH positive during foal inspections in 2009. Out of these 115 affected mares, 77 (67.0%) were included as cases in our study. In 2010, data on 20 additional cases were collected. The 97 cases and 91 controls belonged to 69 owners. Almost three-quarters (71.0%) of the owners had both case(s) and control(s), 23.2% of owners had only case(s), and 5.8% of owners had only control(s). Exposure to *Culicoides* spp. in these latter controls was plausible because of current or previous presence of cases that were unsuitable for our study, for instance, because they were males, which are more often kept indoors and therefore potentially less exposed to *Culicoides* spp.

5.2.4 Blood sample collection and phenotypes

Participating owners were visited by a veterinarian and researcher to take blood samples, to score phenotypes of mares and to conduct an IBH-related questionnaire. Blood collection from Dutch Shetland pony mares was approved by the Board on Animal Ethics and Experiments from Wageningen University (experiment 2009055 and 2010109). Most blood samples were collected in 2009 during September and October (Table 5.1). Blood was collected by jugular venipuncture into two 10-ml EDTA(K_2) Terumo® VenoSafe blood collection tubes per mare. Owners answered questions about the history and management of IBH to increase the information about and reliability of case phenotypes. Questions included the age at onset of IBH, the month during which symptoms began and disappeared, and which preventive and therapeutic measures were taken. During the visit, an experienced veterinarian scored the mare's IBH phenotype using a score sheet for hair loss, hyperkeratosis, scaling, crusting, excoriation, open

wounds and secondary infections on the crest, base of the tail, hindquarters, abdomen, shoulder, neck and head. Severity of symptoms was graded in four categories from mild (category 1) to severe (category 4). However, severity of symptoms was not used during our analysis. To ensure uniform classification, most cases and controls (95.2%) were scored by the same veterinarian (Table 5.1). The remaining five cases and four controls were scored by a second veterinarian.

5.2.5 Genotypes and quality control

DNA was extracted from leucocytes in the blood samples. Lack of degradation of DNA was confirmed by electrophoresis, carried out using a 1-μl sample on 1.5% multi-purpose agarose gels in Tris-borate-EDTA buffer at 120 volts.

Genotypes from all Shetland pony mares were obtained using the Illumina® EquineSNP50 Genotyping BeadChip containing 54,602 SNPs with an average SNP spacing of 43.2 kb.

One homozygote genotype was coded as 0, the heterozygote genotype as 1 and the other homozygote genotype as 2. An additive effect of SNPs was assumed in the regression analysis. Consequently, missing genotypes were centred, that is, they were set to the average value of the genotypes obtained for a single SNP [e.g. in the case of 50 genotypes 0 (AA), 80 genotypes 1 (AB) and 30 genotypes 2 (BB) and for 28 missing genotypes, the missing genotypes were replaced with the average, which was calculated by multiplying genotype frequencies with genotype values ($0.875 = \frac{50}{160} \times 0 + \frac{80}{160} \times 1 + \frac{30}{160} \times 2$)]. The GenABEL package in R (Aulchenko *et al.*, 2007) was used to perform the quality control applying the *check.marker* function. The first step of the iterative procedure of the *check.marker* function excluded SNPs based on per SNP statistics (call rate ≤0.80, minor allele frequency ≤2%, no exclusion based on P-value Hardy-Weinberg equilibrium) using the *summary.snp.data* function. The second step excluded SNPs based on per individual and between individual statistics (call rate ≤0.90, no exclusion based on identity-by-state matrix) using the *perid.summary* function. The procedure was applied recursively until no further SNPs were eliminated. Genotyping failed for 364 SNPs. Monomorphic SNPs (n = 7,957), SNPs with a call rate ≤0.80 (n = 172) and SNPs with minor allele frequency ≤2% (n = 6088) were removed. Of 54,602 SNPs, 40,021 (73.3%) were used in the analyses. Call rate per sample varied from 0.930 to 0.999: 173 Shetland pony mares (92.0%) had a call rate above 0.99, 13 Shetland pony mares (6.9%) had a call rate between 0.95 and 0.99, and only 2 Shetland pony mares (1.1%) had a call rate between 0.90 and 0.95.

After the genome-wide association analysis, quality of SNP clusters and clustering of all significant SNPs was checked using cluster plots, Illumina® GenTrain and GenCall scores. When quality of SNP cluster and clustering was poor (i.e. visual observation of cluster plots, Illumina® GenTrain score <0.55, Illumina® GenCall score <0.7), SNPs were removed (n = 2 out of 26). Based on P-values Hardy-Weinberg equilibrium in controls (all >0.15), no SNPs were removed.

5.2.6 Statistical analysis

To check whether the matching of cases with controls had been successful, the relation between IBH (case or control, binary trait) and matching factors was assessed by means of logistic regression. Fixed effects of withers height category, coat colour, month and year of scoring, region and veterinarian as well as the covariates of the inbreeding coefficient and age of the mare were tested for significance using the LOGISTIC procedure (SAS Institute, Inc.). Factors were tested in both a univariable and multivariable models.

For the genome-wide association analysis, a univariable logistic model was used that fitted SNP genotypes as main effect. The linear predictor (η_i) of the single-SNP model was:

$$\eta_i = \mu + b_1 \times SNP_i$$

where μ = the population mean and b_1 = the allele substitution effect of SNP_i, where *i* = 0, 1 or 2 (for 40,021 SNPs). Estimated allele substitution effects (b_1) were transformed to odds ratios (OR $= e^{b_1}$) for interpretation. An additional analysis was conducted on part of the genome with an extended model, which included a random polygenic effect. The covariance matrix of the random effect was based either on pedigree information or on relationship matrix (= genomic kinship matrix × 2) calculated from SNP genotypes. The additional analysis was conducted to test whether matching of cases with controls on pedigree removed the effect of polygenic variance on SNP effects. For all analyses, we used the !CYCLE option in the ASReml program (Gilmour *et al.*, 2009).

5.2.7 Genomic Kinship

Genomic kinship among Shetland pony mares was computed to determine whether cases and controls differed in kinship (i.e. unsuccessful matching of pedigree). Differences in kinship between cases and controls might result from stratification and thereby lead to false-positive associations. Genomic kinship among mares was

computed using the *ibs* function of the R package GenABEL (Aulchenko *et al.*, 2007) as:

$$f_{i,j} = \sum_k \frac{(x_{i,k} - p_k)(x_{j,k} - p_k)}{(p_k(1 - p_k))}$$

where $f_{i,j}$ = genomic kinship (identity-by-state) matrix between Shetland pony mare i and j, based on k = 38,226 autosomal SNPs (X-chromosome excluded). $x_{i,k}$ or $x_{j,k}$ are the genotypes of the ith or jth Shetland pony mare for SNP k and p_k is the frequency of the allele (top strand).

The genomic kinship matrix was transformed to a distance matrix to perform classical multidimensional scaling (Gower, 1966), which returned the first two principal components. These principal components were plotted to visualise distances between the Shetland pony mares and more specifically between the group of cases and of controls.

5.3 Results

5.3.1 Phenotypes and questionnaire results

Blood samples from 97 cases and 91 controls were collected and included in our analysis. Age at sampling ranged from foal to 22 years (Table 5.1). Age at onset of IBH varied from foal to 15 years, with the majority of the cases (59.8%) starting to show clinical symptoms at the age of 2, 3 or 4 years. For 16.5% of the cases, the year of data collection was the first year with clinical symptoms; for 44.3%, it was the second or third year, and 34.0% of the cases had clinical symptoms for 4 or more years. Most Shetland pony mares developed clinical symptoms during spring (71.1%) and some during summer (20.6%). Often, in 85.1%, symptoms disappeared during autumn. In some cases (7.4%), symptoms disappeared during summer. There was no clear pattern in severity of symptoms of affected mares in consecutive years. Many Shetland pony mares (67.0%) suffered from moderate to severe itch. Crest (99.0%) and base of the tail (82.5%) were mostly affected. In some cases (11.3%), hindquarters were affected. The most frequently observed clinical symptoms were hair loss (97.9%) and thickening/ridged skin (86.6%), whereas crusting (22.7%), open wounds (8.2%) and scaling (7.2%) were observed less frequently. Observed clinical symptoms and most questionnaire results agreed with the typical course of IBH (e.g. Pilsworth and Knottenbelt, 2004). Owners

indicated that the clinical symptoms affected the use of their Shetland pony mares mainly in breeding and showing. Preventive and therapeutic measures (e.g. eczema blanket) were used in 78.4% of the IBH cases.

5.3.2 Success of matching to remove fixed and family effects

Mean age of Shetland pony mares differed significantly (P <0.05) between cases and controls (Table 5.1). The difference in mean age was, however, a result of the experimental setup. For other risk factors, matching was applied. Statistical analysis revealed that these risk factors had no significant effect on IBH prevalence (data not shown). These results indicate that matching of cases with controls was successful in removing difference between cases and controls.

The multi-dimensional scaling plot (Figure 5.1) visualised genetic distances between cases and controls based on the genomic kinship (identity-by-state) matrix. Within the population, subgroups on withers height categories are identified, which is also reflected in Figure 5.1. Shetland pony mares from mini and small withers height categories grouped together (bottom left-hand side Figure 5.1: PC1<0 and PC2<0.05) as did Shetland pony mares from middle and tall withers height categories (bottom right-hand side Figure 5.1: PC1>0 and PC2<0.05). The distribution of mares over the two-dimensional space showed a high degree of

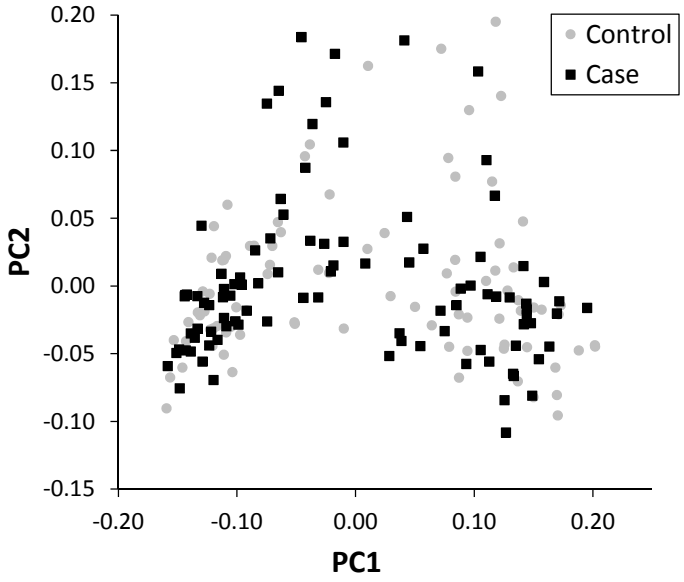

Figure 5.1 Multi-dimensional scaling plot of the distance between Shetland pony mares labelled as case or control. Each point in the plot corresponds to one individual and indicates the distance between mares represented by the first two principal components (PC1 and PC2), which are based on the genomic kinship matrix.

overlap between cases and controls. The multi-dimensional scaling plot based on genomic kinship (Figure 5.1) did not reveal population stratification between cases and controls.

Finally, the heritability for IBH in our sample of cases and controls was estimated with an animal model including either pedigree or genomic-based covariance matrix. Results from both analyses (data not shown) indicated that genetic variance was absent ($h^2 < 0.01$), which reflects that matching of cases with controls on pedigree successfully removed the effect of polygenic variation on IBH.

5.3.3 Genome-wide association study

In total, 24 SNPs on 12 chromosomes were significantly associated [$-\log_{10}$ (P) >2.5] with IBH (Table 5.3, Figure 5.2). The most significant SNP was found on chromosome 27, with the frequency of the unfavourable allele at 0.72 for cases and 0.53 for controls. The odds ratio of allele substitution effect for this SNP was 2.31 (Table 5.3) meaning that the odds of IBH in mares with a heterozygous genotype is increased 2.31 times ($= e^{0.8351}$) compared to mares homozygous for the favourable allele. Further, the odds of IBH in mares homozygous for the unfavourable allele is increased 5.31 times ($= e^{2\times0.8351}$) compared to mares homozygous for the favourable allele. Odds ratios for the significant SNPs were between 1.94 and 5.95 (Table 5.3).

5.4 Discussion

The aims of our study were to identify and quantify genomic associations with IBH in the Dutch Shetland pony population. For this purpose, we applied a genome-wide association study on a matched case-control sample of the Dutch Shetland pony population. To achieve reasonable power to detect genomic regions associated with IBH, we aimed at the collection of samples from 150 cases and 150 controls. Unfortunately, fewer cases than expected were available, which lowered the power to detect associations. We managed to collect blood samples from a large proportion of IBH cases in 2009, but IBH prevalence in 2009 (4.1%) was low compared to years before (8.6% in 2003, 9.5% in 2005 and 8.5% in 2006; Schurink *et al.*, 2009). We collected 20 additional samples in 2010. As our sample size was limited, power to detect QTL with small effect was low and effects of significant QTL tend to be overestimated, which is known as the 'Beavis effect' (Beavis, 1998; Xu, 2003).

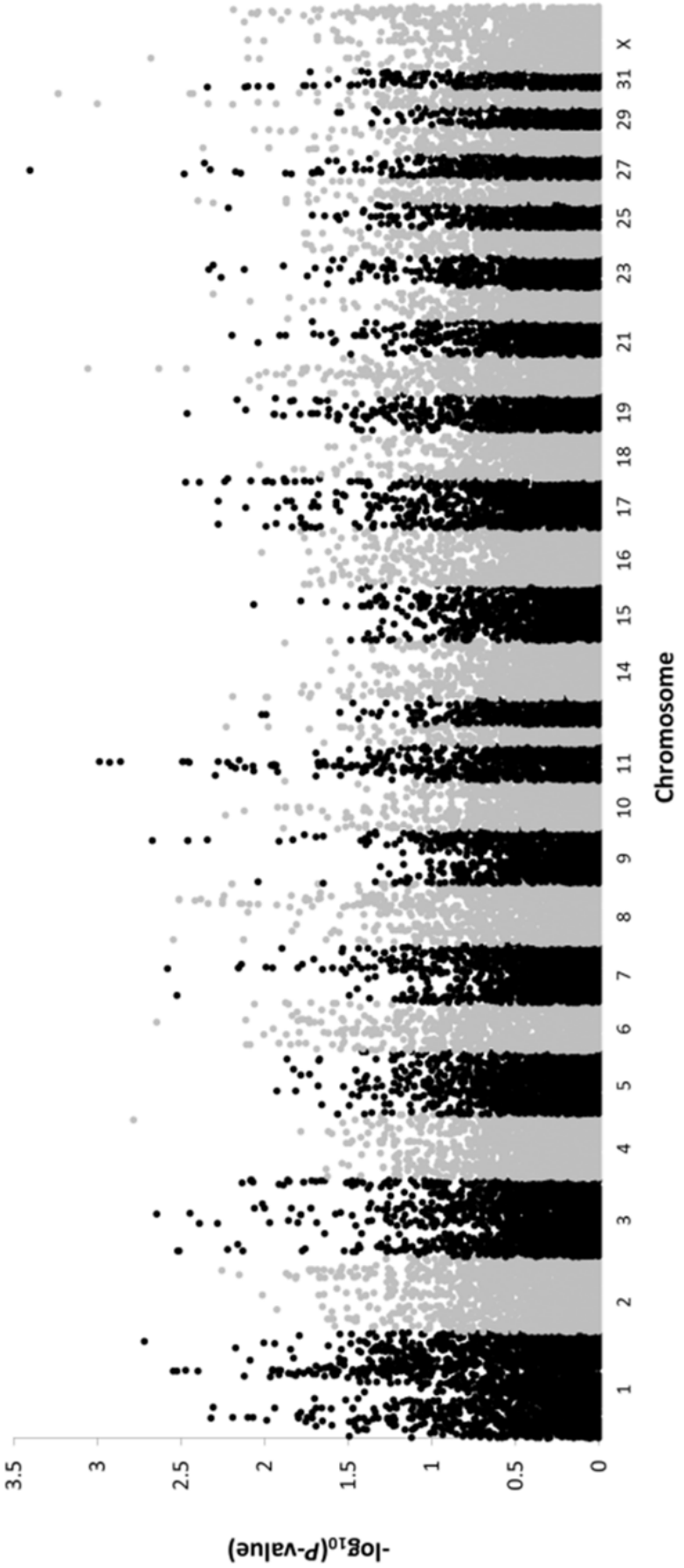

Figure 5.2 Genome-wide association plot of insect bite hypersensitivity in Dutch Shetland pony mares. The $-\log_{10}$(P-value) is plotted against the chromosomal location for each SNP tested across all chromosomes. Chromosome number is indicated on the x-axis, where the chromosomes are plotted in alternating black and grey for clarity.

Table 5.3 Single nucleotide polymorphisms (SNPs) showing most significant association with insect bite hypersensitivity in Dutch Shetland pony mares using a single-SNP model.

					Allele frequency[c]	
SNP name	ECA[a]	Position (bp)	P-value	OR (95% CI)[b]	Cases	Controls[d]
BIEC2-50714[e]	1	116,022,321	0.0030	2.31 (1.34-3.98)	0.27	0.14
BIEC2-50725[e]	1	116,037,195	0.0029	2.53 (1.39-4.63)	0.22	0.09
BIEC2-81773[f]	1	171,091,112	0.0019	5.95 (1.96-18.1)	0.98	0.89
BIEC2-771602[e]	3	8,370,895	0.0030	1.96 (1.26-3.05)	0.59	0.43
BIEC2-771605[e,g]	3	8,392,689	0.0031	2.00 (1.27-3.15)	0.57	0.42
BIEC2-786998[g]	3	64,632,334	0.0023	3.71 (1.62-8.52)	0.14	0.04
BIEC2-877664	4	99,194,126	0.0017	2.94 (1.52-5.69)	0.20	0.08
BIEC2-953011	6	47,258,572	0.0023	2.18 (1.33-3.57)	0.77	0.63
BIEC2-980932	7	13,582,164	0.0030	2.08 (1.29-3.35)	0.82	0.68
BIEC2-1004427	7	61,379,694	0.0026	2.31 (1.35-3.97)	0.29	0.16
BIEC2-1027098[g]	8	8,138,630	0.0029	2.64 (1.41-4.95)	0.21	0.10
BIEC2-1062146[e]	8	70,132,295	0.0031	2.05 (1.28-3.28)	0.80	0.66
BIEC2-1062154[e]	8	70,132,806	0.0031	2.05 (1.28-3.28)	0.80	0.66
BIEC2-1102005	9	66,153,825	0.0021	2.04 (1.30-3.20)	0.63	0.47
BIEC2-149137	11	32,010,755	0.0012	2.48 (1.44-4.25)	0.28	0.13
BIEC2-149356[e]	11	32,753,122	0.0010	2.93 (1.56-5.51)	0.22	0.09
BIEC2-149592[e]	11	33,040,164	0.0014	2.90 (1.53-5.52)	0.21	0.09
BIEC2-532511	20	41,520,518	0.0023	2.01 (1.29-3.13)	0.44	0.28
BIEC2-532524	20	41,531,646	0.0009	2.17 (1.38-3.39)	0.45	0.27
BIEC2-705454	27	13,198,799	0.0004	2.31 (1.46-3.63)	0.72	0.53
BIEC2-814955	30	4,484,018	0.0010	2.21 (1.39-3.52)	0.75	0.58
BIEC2-825651[e,f]	30	20,973,099	0.0006	5.43 (2.10-14.0)	0.97	0.87
BIEC2-825652[e,f]	30	20,973,284	0.0006	5.43 (2.10-14.0)	0.97	0.87
BIEC2-1114599	X	23,168,684	0.0021	1.94 (1.28-2.94)	0.53	0.36

[a]*Equus caballus* autosome.

[b]Odds ratio of allele substitution effect of unfavourable allele calculated as e^{b_1}; 95% confidence interval between parentheses.

[c]Allele frequency of unfavourable allele.

[d]No deviations from Hardy-Weinberg equilibrium (all P >0.15) were detected in controls.

[e]Linkage disequilibrium (r^2) between SNPs on same chromosome is >0.8.

[f]Only two out of three genotypes were present (i.e. homozygotes for one allele and heterozygotes).

[g]Homozygote for one allele absent in controls.

Genomic associations with IBH are potentially confounded by other effects relevant for IBH (e.g. region, withers height category). However, these effects were nullified in our study by using a matched case-control design. Age of the mare was significantly associated with IBH but was not included in the analysis of genomic associations as it was introduced by the study design and potentially could lower the power to detect associations between IBH and genotypes. Data comprised 89

paternal half-sibs (47.3% of the data). Previous genetic analysis of IBH in the Shetland pony population revealed significant genetic variance corresponding to an estimated heritability of 0.24 (Schurink *et al.*, 2009). A similar genetic analysis of our case-control sample revealed no significant genetic variance. Also, the multi-dimensional scaling plot based on the genomic kinship (Figure 5.1) showed a high degree of overlap between cases and controls over the two-dimensional space. These results indicate that cases and controls had been successfully matched to minimise effects of population stratification and unwanted spurious associations in the genome-wide association study.

Misclassification of case or control phenotypes – misclassification bias (e.g. McCarthy *et al.*, 2008) – is expected to reduce allele frequency differences between cases and controls and therefore reduces power of the association study (e.g. Edwards *et al.*, 2005; Pearson and Manolio, 2008). To diminish potential misclassification bias, Dutch Shetland pony mares were selected according to a strict protocol.

Candidate gene approach or genome-wide association studies on IBH in horses are limited. *Serine peptidase inhibitor, Kazal type 5* (*SPINK5*) in humans has been related to disorders with equivalent clinical symptoms as IBH. Andersson *et al.* (2009), therefore, considered *SPINK5* on ECA14 to be a candidate gene, but found that it was not associated with IBH in Swedish-born Icelandic horses. Wagner *et al.* (2001) identified polymorphisms in the *immunoglobulin heavy constant epsilon* (*IGHE*) gene on ECA24 that encodes for the heavy chain constant region domains of equine IgE, which mediates IBH (Wagner *et al.*, 2006). Wagner *et al.* (2001) suggested that polymorphisms in the *IGHE* gene might impose genetic variability to allergic diseases in horses, but they did not study associations. Hořín *et al.* (2004) carried out an association study and identified and tested polymorphic markers (both microsatellites and SNPs) in various immune response-related genes to identify associations with two important horse infections: *R. equi* and *L. intracellularis*. Alleles of, for instance, the microsatellite locus *HMS01* were significantly associated with *R. equi* infection. Immune response-related genes [e.g. *IL10* (*interleukin 10*)] were found within the associated regions, although fine-mapping is needed to assign candidate genes (Hořín *et al.*, 2004). Locus *HMS01* on ECA15 previously has been identified to be associated with IBH (Marti *et al.* 2005 in Chowdhary and Raudsepp, 2008). Above-mentioned candidate genes and regions associated with IBH, however, did not coincide with the genomic regions associated with IBH in our study.

The equine major histocompatibility complex (MHC), or ELA, recognizes many foreign molecules and thereby evokes an immune response (Bailey *et al.*, 2000).

Equine ELA class I and II regions are positioned on ECA20 and were associated with allergen-specific immunoglobulin E (IgE) levels against two moulds (Eder *et al.*, 2001) and IBH (Halldórsdóttir *et al.*, 1991; Marti *et al.*, 1992; Andersson *et al.*, 2012). Eder *et al.* (2001) investigated associations between ELA class I antigens and allergen-specific IgE levels against two moulds and found that horses carrying ELA A1 had a significantly higher IgE level against *Aspergillus f.* and that horses carrying ELA A14 had significantly lower IgE levels against both *Alternaria a.* and *Aspergillus f.* Moreover, serological research on ELA class II antigens identified a significant difference in distribution of the Be 8 antigen between IBH cases and controls (Halldórsdóttir *et al.*, 1991). Also, the W23 antigen segregated with IBH predisposition in specific families (Marti *et al.*, 1992). Recently, genomic research identified an association between the ELA class II region and IBH in two distinct horse breeds (Andersson *et al.*, 2012). Although we found associations with IBH on ECA20 (Table 5.3), the identified genomic region was approximately 8 Mb away from ELA regions. Therefore, we cannot confirm nor exclude the impact of ELA on IBH. This requires a closer investigation of ELA regions.

From our study, using visual inspection of the Manhattan plot (Figure 5.2) to identify interesting genomic regions, it appeared that 24 SNPs on 12 chromosomes were associated with IBH [$-\log_{10}$ (P) >2.5]. After calculating the false discovery rate using *q*-values (Storey and Tibshirani, 2003), no SNP exceeded a false discovery rate of 0.5 (data not shown). Odds ratios of allele substitution effects of the unfavourable allele were between 1.94 and 5.95. Population attributable fractions (PAF), which indicate the reduction in IBH prevalence when eliminating the unfavourable allele, were calculated following Bertram *et al.* (2007). Based on the odds ratios and allele frequency of the unfavourable allele in controls, PAFs varied from 0.11 to 0.42. Hence, the IBH prevalence in the Dutch Shetland pony population, being 7.6% (Schurink *et al.*, 2010), could be reduced to values between 4.4 and 6.8% (depending on PAF per SNP) when the unfavourable allele is eliminated. However, this reduction in prevalence is overestimated as the odds ratio overestimates relative risk (Schmidt and Kohlman, 2008). Second, the allele frequency of the unfavourable allele in the population estimated from our controls might not represent the real allele frequency in the Dutch Shetland pony population.

Genomic selection using only significant markers or all markers could increase the efficiency of breeding for decreased IBH prevalence. The increased efficiency results from increased accuracies of estimated breeding values and decreased generation interval (e.g. Meuwissen *et al.*, 2001; Hayes and Goddard, 2010)

compared to traditional breeding using own performance or progeny information. Own performance for IBH depends highly on exposure to *Culicoides* spp. and can be recorded only from an age of 3 years. Progeny testing of sires will improve accuracy of breeding values but will increase generation interval, as sufficient progeny information will be available only late in life. Genomic selection can be applied at a young age because it requires collection of only marker genotypes but not of phenotypic information on the selection candidate or its progeny. Phenotypic information already collected in the Shetland pony population is exploited through its genomic association to markers.

Horses from various breeds in many countries worldwide are affected with IBH and prevalence varies between breeds, even within a similar environment. Breed differences in susceptibility to IBH are expected, although a common genetic background is anticipated. Genome-wide association analyses of IBH in additional samples from the Dutch Shetland pony population or in samples from other horse breeds are needed to validate the identified associations and to narrow down the genomic regions.

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6

Genome-wide association study of insect bite hypersensitivity in two horse populations in the Netherlands

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Abstract

Insect bite hypersensitivity (IBH) is a common allergic disease in horse populations worldwide. Insect bite hypersensitivity is affected by both environmental and genetic factors. However, little is known about genes contributing to the genetic variance associated with IBH. Therefore, the aim of our study was to identify and quantify genomic associations with IBH in Shetland pony mares and Icelandic horses in the Netherlands. Data on 200 Shetland pony mares and 146 Icelandic horses were collected according to a matched case-control design. Cases and controls were matched on various factors (e.g. region, sire) to minimize effects of population stratification. Breed-specific genome-wide association studies were performed using 70k SNP genotypes. Bayesian variable selection method Bayes-C with a threshold model implemented in GenSel software was applied. A 1 Mb non-overlapping window approach that accumulated contributions of adjacent SNP was used to identify associated genomic regions. The percentage of variance explained by all SNP was 13% in Shetland pony mares and 28% in Icelandic horses. The 20 non-overlapping windows explaining the largest percentages of genetic variance were found on nine chromosomes in Shetland pony mares and on 14 chromosomes in Icelandic horses. Overlap in identified associated genomic regions between breeds would suggest interesting candidate regions to follow-up on. Such regions common to both breeds (within 15 Mb) were found on chromosomes 3, 7, 11, 20 and 23. Positional candidate genes within 2 Mb from the associated windows were identified on chromosome 20 in both breeds. Candidate genes are within the equine lymphocyte antigen class II region, which evokes an immune response by recognizing many foreign molecules. The genome-wide association study identified several genomic regions associated with IBH in Shetland pony mares and Icelandic horses. On chromosome 20, associated genomic regions in both breeds were within 2 Mb from the equine lymphocyte antigen class II region. Increased knowledge on IBH associated genes will contribute to our understanding of its biology, enabling more efficient selection, therapy and prevention to decrease IBH prevalence.

Key words: Bayesian variable selection, insect bite hypersensitivity, genome-wide association study, matched case-control design, multi-SNP model

6.1 Introduction

Insect bite hypersensitivity (IBH) is a common allergic skin disease in various horse breeds found throughout the world, and results from bites of *Culicoides* spp. Sensitive horses develop a severe itch, which results in self-inflicted trauma and severely affected horses sometimes need to be euthanized (Gortel, 1998). Welfare of affected horses is therefore reduced. No cure is available, and methods to prevent or reduce clinical symptoms often require dedication from the owner and greatly differ in efficiency (e.g. Gortel, 1998; Meiswinkel *et al.*, 2000; de Raat *et al.*, 2008; Papadopoulos *et al.*, 2010; van den Boom *et al.*, 2011). Owners of affected horses incur costs related to preventive or curative methods and veterinary consultation. Moreover, the commercial value of affected horses is reduced and use of affected horses can be restricted due to discomfort and disfiguration (Gortel, 1998).

Insect bite hypersensitivity is a multi-factorial disorder that is affected by environmental and genetic factors. Environmental factors are, among others, related to *Culicoides* spp. density. Partial genetic control has been confirmed in various horse breeds (Eriksson *et al.*, 2008; Schurink *et al.*, 2009; Schurink *et al.*, 2011). Monogenic inheritance of sensitivity to IBH has been rejected by segregation analysis (Unkel *et al.*, 1987), which showed a polygenic mode of inheritance. However, little is known on the genes contributing to genetic variance. Genomic research on IBH using a candidate gene approach or genome-wide association study (GWAS) has been limited. Using a candidate gene approach, Andersson *et al.* (2012) showed that variants within the major histocompatibility complex (MHC) class II region are associated with IBH sensitivity. The same allele (*COR112*:274) increased IBH sensitivity in both Swedish-born Icelandic horses (odds ratio = 4.19) and Exmoor ponies (odds ratio = 1.48). Moreover, homozygosity across the MHC class II region increased IBH sensitivity in both breeds. Serological research on IBH has also shown a significant difference in the distribution of specific MHC antigens between cases and controls (Halldórsdóttir *et al.*, 1991; Marti *et al.*, 1992). The MHC genes in the horse, known as equine lymphocyte antigen (ELA) genes, are located on horse chromosome (*Equus caballus*) ECA20 and their resulting protein structures recognize many foreign molecules, thereby evoking an immune response (Bailey *et al.*, 2000).

Using GWAS, Schurink *et al.* (in press) found associations between ECA20 and IBH. The identified genomic region was approximately 8 Mb from the MHC region and was poorly covered in single nucleotide polymorphisms (SNP) from the marker panel. Schurink *et al.* (in press) identified 24 SNP on 12 chromosomes in Shetland

pony mares associated with IBH sensitivity [$-\log_{10}(P)$ >2.5]. Insect bite hypersensitivity is observed in many horse breeds throughout the world and could have common genetic components across breeds. Across-breed analyses could facilitate fine-mapping by reducing the length of the associated genomic regions, since haplotypes shared across breeds are expected to be shorter than within-breed haplotypes (e.g. Karlsson and Lindblad-Toh, 2008; Hayes and Goddard, 2010).

The aim of our study was to expand these findings through identification and quantification of genomic associations with IBH using phenotypic and SNP information from Shetland pony mares and Icelandic horses in the Netherlands. Knowledge of genomic regions associated with IBH will contribute to our understanding of its biology, enabling more efficient selection, therapy and prevention in order to decrease IBH prevalence.

6.2 Methods

6.2.1 Animals and phenotypes

Cases were defined as individuals showing clinical IBH symptoms, while controls were free of symptoms despite exposure to *Culicoides* spp. Selection of cases and controls was described in detail by Schurink *et al.* (in press), and cases and controls were matched on various factors to minimize effects of population stratification. Shetland pony mares were recruited through routine inspections in 2009 and through publications by the studbook in their magazine and on their website in 2010. Shetland pony mares were matched on withers height category, coat colour, location and sire. Icelandic horses were recruited in 2010 through publications on various equine related websites and were matched on coat colour, location, sex, importation from Iceland (yes/no) and sire. Age at onset is generally between 2 and 4 years-of-age (e.g. van den Boom *et al.*, 2008). Therefore, controls were required to be at least 4 years-of-age and to have been at least one year at risk for developing symptoms. Proximity to a case was required to ensure exposure to *Culicoides* spp. and thereby increase reliability of phenotypes on controls. Paternal half-sibs were sought to minimize population stratification due to pedigree. The data set (Table 6.1) contained 200 Shetland pony mares and 146 Icelandic horses. The same Shetland pony mares analysed by Schurink *et al.* (in press) were included in our study, although 70k genotype data were available, since the mares were re-genotyped.

Table 6.1 Distribution of characteristics of Shetland pony mares and Icelandic horses for cases and controls.

	Shetland pony mares			Icelandic horses		
Trait	Cases	Controls	Total	Cases	Controls	Total
Number of animals	103	97	200	73	73	146
Year of scoring						
2009	80.6	80.4	80.5	-	-	-
2010	19.4	19.6	19.5	100.0	100.0	100.0
Month of scoring						
September	50.5	53.6	52.0	30.1	39.7	34.9
October	45.6	43.3	44.5	69.9	60.3	65.1
November	3.9	3.1	3.5	-	-	-
Veterinarian						
1	94.2	95.9	95.0	100.0	100.0	100.0
2	5.8	4.1	5.0	-	-	-
Pedigree						
Number of sires	84	86	129	57	61	95
Number of dams	100	93	187	68	67	126
Age, years						
Mean (sd)	7.1 (4.5)	8.3 (4.4)	7.7 (4.5)	13.1 (6.0)	12.6 (5.9)	12.8 (5.9)
Range	0 – 23	4 – 22	0 – 23	4 – 29	4 – 35	4 – 35
Sex						
Female	100.0	100.0	100.0	69.9	58.9	64.4
Male	-	-	-	30.1	41.1	35.6
WHC[b]						
Mini	27.2	24.7	26.0			N/A[a]
Small	16.5	18.6	17.5			
Middle	31.1	27.8	29.5			
Tall	25.2	28.9	27.0			
Import[c]						
Yes			N/A[a]	23.3	2.7	13.0
No				76.7	97.3	87.0
Coat colour						
Bay	4.9	4.1	4.5	15.0	9.6	12.3
Black	49.5	53.6	51.5	21.9	15.1	18.4
Black paint	5.8	4.1	5.0	2.7	4.1	3.4
Chestnut	25.2	23.7	24.5	11.0	19.2	15.1
Chestnut paint	7.8	6.2	7.0	-	2.7	1.4
Other	6.8	8.3	7.5	38.4	47.9	43.2
Silver dapple	-	-	-	11.0	1.4	6.2

[a]N/A = not applicable.

[b]WHC = withers height category.

[c]Import indicates whether an Icelandic horse has been imported from Iceland or not.

Participating owners were visited by an experienced veterinarian and researcher to take blood samples, score phenotypes and conduct an IBH related questionnaire (more details in Schurink *et al.*, in press). All Icelandic horses and most Shetland pony mares (95.0%) were scored by the same veterinarian (Table 6.1) to ensure uniform classification. Blood sample collection from Shetland pony mares and Icelandic horses was approved by the Board on Animal Ethics and Experiments from Wageningen University (experiments 2009055 and 2010109).

6.2.2 Data

Shetland pony mare data contained 103 cases and 97 controls collected in autumn 2009 or 2010 (Table 6.1). Data contained half-sib mares (50.0% of the data) descending from 41 sires with both case(s) and control(s) among their offspring, and mares (50.0% of the data) descending from 88 sires with only case(s) or control(s) among their offspring. Mares were located on 73 premises. The number of mares per premise ranged from 1 (23.3% of all premises) to 9 (2.7%) and the mean number of mares per premise was 2.7.

Icelandic horse data contained 73 cases and 73 controls collected in autumn 2010 (Table 6.1). It contained both females (64.4%) and males (i.e. geldings and stallions; 35.6%) (Table 6.1). In total, 13.0% of Icelandic horses were imported from Iceland and 87.0% were born in Europe (mainly the Netherlands) (Table 6.1). Data contained half-sib horses (45.2% of the data) descending from 23 sires with both case(s) and control(s) among their offspring, and horses (54.8% of the data) descending from 72 sires with only case(s) or control(s) among their offspring. Horses were located on 31 premises. The number of horses per premise ranged from 1 (19.4% of all premises) to 14 (3.2%) and the mean number of horses per premise was 4.7.

6.2.3 Genotyping and quality control

Genotypes from all Shetland pony mares and Icelandic horses were obtained using the equine HD chip (Illumina Inc., San Diego, CA) containing 65,157 SNP. Those SNP with a call-rate <90% or minor allele frequency ≤0.02 were excluded from the data. Call-rate per animal was considered sufficient (>90%) for all animals. The majority (319 out of 346) of animals had a call-rate greater than 99%. After breed-specific quality control (applying the same quality control to each breed separately), the Shetland pony mare data contained 46,888 SNP and the Icelandic horse data contained 51,453 SNP.

6.2.4 Population stratification analysis

Cases and controls were matched on various factors to minimize effects of population stratification and thereby reduce possible spurious associations. To test whether matching of cases and controls in Shetland pony mares and Icelandic horses was successful, the relation between IBH (case or control, binary phenotype) and matching factors was assessed in univariable and multivariable models using the LOGISTIC procedure incorporated in SAS 9.2© software (SAS Institute Inc., Cary, NC). Fixed effects of withers height category, coat colour, sex, import from Iceland, veterinarian and month and year of scoring, and the covariate of age of the animal were tested for significance.

Similar genomic kinship within and across cases and controls indicates successful matching on pedigree. Breed-specific genomic kinship among animals was therefore computed using the *ibs* function of the R package GenABEL (Aulchenko *et al.*, 2007) as:

$$f_{i,j} = \sum_k \frac{(x_{i,k} - p_k)(x_{j,k} - p_k)}{(p_k(1-p_k))},$$

where $f_{i,j}$ is the genomic kinship (identity-by-state) between animal i and j, based on k = 48,810 autosomal SNP in Icelandic horses (SNP with call-rate <90%, monomorphic SNP and SNP on the X chromosome excluded) and 44,576 autosomal SNP in Shetland pony mares; $x_{i,k}$ or $x_{j,k}$ are the genotypes (coded as 0, ½, 1) of the i^{th} or j^{th} animal for SNP k and p_k is the frequency of the allele (top strand). The genomic kinship matrix was transformed to a distance matrix to perform classical multidimensional scaling (Gower, 1966), which returned the first two principal components. The principal components for each breed were plotted to visualize distances between animals and more specifically between cases and controls. Further, Icelandic horses were categorized into 'imported from Iceland' or 'born in Europe' to see whether their genetic background differed.

6.2.5 Genome-wide association study

Breed-specific GWAS were performed using genotypes from the same marker panel but the number of SNP after quality control differed between Shetland pony mares (n = 46,888) and Icelandic horses (n = 51,453). The Bayesian variable selection method Bayes-C with a threshold model, described by Kizilkaya *et al.* (2010) and implemented in the GenSel software (http://bigs.ansci.iastate.edu/) was used to identify and quantify genomic regions associated with IBH. Method

Bayes-C assumes a common variance for all SNP in the model and is less sensitive to the prior for genetic variance (e.g. Fernando and Garrick, 2008; Fan *et al.*, 2011; Onteru *et al.*, 2011) compared to Bayes-B as described by Meuwissen *et al.* (2001). Method Bayes-C fits all SNP simultaneously using a mixture threshold model and assuming additive SNP effects:

$$\eta = \mu + \sum_{j=1}^{K} Z_j u_j \delta_j$$

where η is the linear predictor that is related to observed IBH phenotypes (case/control) through a probit link function and was sampled during each iteration from a normal distribution that comprises the liability scale corresponding to the observed threshold score following Sorensen *et al.* (1995); μ is an overall mean; K is the number of SNP; Z_j is the column vector representing the genotype covariate at SNP j (input as AA = -10, AB = 0, BB = 10 with missing genotypes set to the average value of the particular SNP in the data set); u_j is the random allele substitution effect of SNP j, and δ_j is a random 0/1 variable indicating the absence (with probability π) or presence (with probability $1-\pi$) of SNP j in the model.

In our analyses, π was set to 0.999, resulting in roughly 30 to 70 SNP being included in the model in any particular iteration. Fewer SNP than individuals were fitted in any iteration to decrease the risk of overfitting the data, and previous work (Schurink *et al.*, in press) showed that a limited number of SNP reach significance level. The allele substitution effect for SNP j (u_j) was assumed normally distributed $\sim N\left(0, \sigma_u^2\right)$ conditional on σ_u^2 when SNP j was included in the model ($\delta_j = 1$), but u_j was 0 when $\delta_j = 0$. Variance σ_u^2 was assumed to follow a scaled inverse chi-square distribution with $v_u = 4$ degrees of freedom and scale parameter S_u^2, which was derived from the additive genetic variance as $\frac{\sigma_a^2}{K(1-\pi)2\overline{p}\,\overline{q}}$ according to Gianola *et al.* (2009) and Kizilkaya *et al.* (2010). The prior of σ_u^2 was derived from the heritability of IBH on the liability scale (= 24%), as estimated in a pedigree-based population genetic analysis by Schurink *et al.* (2009). Residual variance σ_e^2 is not identifiable and was set to 1 and not sampled.

Table 6.2 Questionnaire results (%) from Shetland pony mare and Icelandic horse cases of insect bite hypersensitivity.

Trait	Shetland pony mares	Icelandic horses
Age at onset		
Younger than 2 yr	9.7	1.7
2 to 5 yr	62.2	50.8
6 to 10 yr	15.5	22.0
11 yr or older	1.9	10.2
Unknown	10.7	15.3
Duration IBH		
1 yr	17.5	-
2 yr	22.3	-
3 yr or more	55.3	100.0
Unknown	4.9	-
Onset of symptoms		
Spring	68.0	81.3
Summer	25.2	10.2
Autumn	1.0	-
Unknown	5.8	8.5
Disappearance symptoms		
Summer	7.0	-
Autumn	81.3	89.8
Winter	1.0	1.7
Chronic	2.9	-
Unknown	7.8	8.5
Severity of symptoms over years		
Increases	8.7	1.7
Decreases	6.8	11.9
Remains equal	46.6	16.9
Varies	21.4	59.3
Unknown	16.5	10.2
Severity of itch		
Mild	25.2	44.0
Moderate	32.0	37.3
Severe	37.0	15.3
Unknown	5.8	3.4
Preventive or curative measures		
Yes	82.5	98.3
No	17.5	1.7
Applied measures[a]		
Eczema blankets	27.2	86.4
Local treatment with oil or cream	69.9	76.3
Insecticide	21.4	42.4
Related to nutrition	2.9	28.8
Stabling	3.9	8.5

[a]Expressed as percentage of total number of cases per measure. In several cases more measures were applied, sum of percentages is therefore >100%.

Sampling of effects is described in more detail by Kizilkaya *et al.* (2010). A total of 200,000 Markov chain Monte Carlo (MCMC) iterations were run, with a burn-in period of 20,000 iterations.

Model frequency, i.e. the proportion of total post burn-in iterations in which a particular SNP was included in the model, was used as evidence for an associated SNP. However, if consecutive SNP are in high linkage disequilibrium (LD) with a particular quantitative trait locus (QTL), effects and model frequencies may be distributed across those SNP, and effects and model frequencies of individual SNP will completely capture the effects of the QTL. Thus, a window approach, which accumulates effects of adjacent SNP, was used to better identify genomic regions associated with QTL (Sun *et al.*, 2011).

The approach described by Wolc *et al.* (2012) and implemented in version 4.0 of the GenSel software (http://bigs.ansci.iastate.edu/) was used to identify associated windows (i.e. genomic regions). For this purpose, physical map order (build EquCab2.0) was used to allocate SNP to consecutive non-overlapping 1 Mb windows (n = 2,376) and the posterior distribution of the percentage of genomic variance explained by each of these windows was derived. For this purpose, the variance of genomic breeding values for each window (= window genomic variance) was computed among individuals for every 100^{th} iteration of the MCMC chain based on the marker effects sampled in that iteration. Window genomic variance was divided by genomic variance explained (sum of all SNP) across the genome in that particular iteration to determine the percentage of genomic variance explained by the window. The resulting posterior distribution of the % variance of each window was used for testing. The posterior distribution included results from iterations that excluded the window (or SNP) from the model. Window genomic variance greater than 0.04% [i.e. the expected percentage of variance explained by each window in an infinitesimal model ($\frac{1}{2{,}376} \times 100$)], was used as a threshold to declare regions that explained more variance than expected.

6.3 Results

6.3.1 Phenotypes and questionnaire results

Questionnaire results from Shetland pony mare and Icelandic horse cases are shown in Table 6.2. In Icelandic horses, questionnaire data from 14 out of 73 cases was missing. The observed clinical symptoms of IBH in all cases are summarized in

Table 6.3 Insect bite hypersensitivity symptoms on various locations in Shetland pony mare and Icelandic horse cases[a].

	Shetland pony mare cases	Icelandic horse cases
Clinical symptoms		
Hair loss	97.1	97.3
Thickening of skin	86.4	97.3
Crusting	25.2	23.3
Scaling	8.7	5.5
Open wounds	8.7	1.4
Affected location		
Crest	99.0	98.6
Base of the tail	83.5	83.6
Hindquarters	21.4	-
Head	3.9	16.4
Abdomen	1.0	11.0
Other	3.9	-

[a]Clinical symptoms were scored by an experienced veterinarian and are presented as the percentage of cases with this particular clinical symptom; in total, 103 Shetland pony mare cases and 73 Icelandic horse cases were scored.

Table 6.3. Severity of itch was lower in Icelandic horse cases compared with Shetland pony mare cases (Table 6.2), probably because eczema blankets (a preventive measure) were used in many Icelandic horse cases. Preventive or curative measures were applied more often for Icelandic horse cases than for Shetland pony mare cases (Table 6.2) and more measures per case were applied to Icelandic horse cases compared to Shetland pony mare cases. Owners of cases replied that they experienced negative effects of IBH, as it reduces equine welfare, requires much time and limits rideability and marketability. For both Icelandic horse and Shetland pony mares, questionnaire results and observed clinical symptoms agreed with the typical course of IBH (e.g. Pilsworth and Knottenbelt, 2004).

6.3.2 Population stratification analysis

Matching of Shetland pony mares to minimize effects of population stratification was successful, as none of the matching factors had a significant effect on IBH ($P < 0.05$). Analysis of matching factors in Icelandic horses indicated that import from Iceland ($P = 0.002$) had a significant effect on IBH.

To test whether matching of cases and controls based on sire was successful, breed-specific genomic kinship among animals was computed based on identity-by-state of SNP genotypes. Figures 6.1 and 6.2 show the first two principal

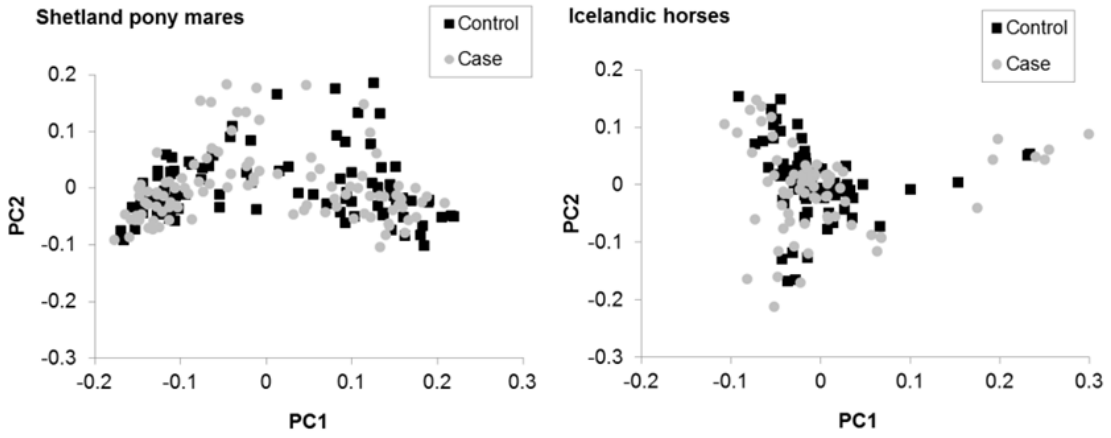

Figure 6.1 Multi-dimensional scaling plots of the genetic distance between animals in Shetland pony mares and Icelandic horses. Each point corresponds to one animal and indicates the distance between animals represented by the first two principal components (PC1 and PC2), based on the genomic kinship matrices.

components of the transformed breed-specific kinship matrices to visualize genetic distances between animals. The multidimensional scaling plots showed a high degree of overlap between cases and controls in both Shetland pony mares and Icelandic horses (Figures 6.1). Effects of population stratification due to pedigree are therefore limited.

Two imported approved stallion cases were more distant to the other Icelandic horses in our dataset, although the imported Icelandic horses seemed to originate from a similar genetic background (Figure 6.2). However, imported Icelandic horses (n = 17 cases and n = 2 controls) were removed from the analyses due to less successful matching of Icelandic horses on import status (Table 6.1 and P = 0.002 for import status). The final Icelandic horse data therefore included 56 cases and 71 controls and were all born in Europe.

6.3.3 Genome-wide association study

In Shetland pony mares, 13% of variance was explained by all SNP, which is lower than the pedigree-based estimate of heritability of IBH on the liability scale (24%, se = 6%) in Shetland pony mares in the Netherlands (Schurink *et al.*, 2009). The 20 non-overlapping windows that explained the largest percentages of genetic variance were located on nine chromosomes (Table 6.4). The percentage of genetic variance explained by the top 20 associated windows ranged from 0.62 to 0.14% (Table 6.4) and was highest for the window on chromosome 20 position 35 Mb

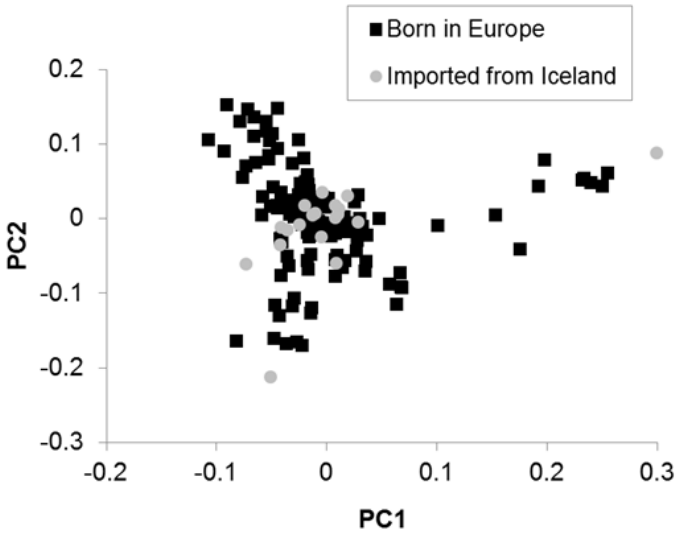

Figure 6.2 Multi-dimensional scaling plot of the genetic distance between imported Icelandic horses and Icelandic horses born in Europe. Each point corresponds to one animal and indicates the distance between animals represented by the first two principal components (PC1 and PC2), based on the genomic kinship matrices.

(Table 6.4). In 2.7 to 5.3% of iterations of the MCMC (Table 6.4), the percentage of variance explained by a window exceeded the expected percentage of variance explained (i.e. 0.04%). For each of the top 20 associated windows, the SNP with the highest model frequency is presented in Table 6.4, including the frequency of the unfavourable allele in cases and controls.

In Icelandic horses born in Europe, 28% of variance was explained by all SNP, which is equal to the pedigree-based estimate of heritability of IBH on the liability scale (27%, se = 17%) in Swedish-born Icelandic horses (Eriksson *et al.*, 2008). The 20 windows explaining the largest percentages of genetic variance were located on 14 chromosomes (Table 6.5). The percentage of genetic variance explained by the top 20 associated windows ranged from 0.66 to 0.14% (Table 6.5) and was highest for the window on chromosome X position 59 Mb (Table 6.5). In 2.2 to 7.9% of iterations of the MCMC (Table 6.5), the percentage of variance explained by a window exceeded the expected percentage of variance explained (i.e. 0.04%). For each of the top 20 associated windows, the SNP with the highest model frequency is presented in Table 6.5.

A comparison of associated genomic regions in Shetland pony mares and Icelandic horses (using the percentage of genetic variance explained by 1 Mb non-overlapping windows in the breed-specific GWAS) is depicted in Figure 6.3. An overlap in the top 20 associated genomic regions (≥0.14% of genomic variance

explained) was found on chromosomes 3, 7, 11, 20 and 23 (within 5 to 15 Mb), and represent the most promising candidate regions to follow-up on.

6.4 Discussion

The aim of this study was to identify and quantify genomic regions associated with IBH in Shetland pony mares and Icelandic horses in the Netherlands. Breed-specific GWAS were performed and overlapping associated genomic regions (within 15 Mb or less) were identified in both breeds.

6.4.1 Population stratification analysis

Data were gathered according to a matched case-control design to limit unwanted spurious associations due to population stratification, which might be caused by confounding of 'the trait of interest' with pedigree and other relevant (e.g. environmental) effects (e.g. Hirschhorn and Daly, 2005). Population stratification due to pedigree was minimized in both breeds by including paternal half-sib pairs. The multidimensional scaling plots based on breed-specific genomic kinship showed a high degree of overlap between cases and controls (Figure 6.1). Also, the Bayes method accounts for population stratification due to pedigree by fitting all SNP simultaneously (e.g. Toosi *et al.*, 2010). In Shetland pony mares, confounding of IBH with relevant effects such as region and withers height category was negligible, since the analysis revealed no significant association between IBH and these effects. In Icelandic horses, importation from Iceland had a significant effect on IBH (P = 0.002). Our results showed (Figure 6.2) that differences in genetic background between imported Icelandic horses and Icelandic horses born in Europe were limited, which agrees with Broström *et al.* (1987) and Andersson *et al.* (2012). However, *Culicoides* spp. is absent in Iceland and consequently IBH is not observed (e.g. Marti *et al.*, 2008). Increased environmental pressure after export and lack of exposure to *Culicoides* spp. before export are suggested to result in increased incidence and more severe cases after export (e.g. Broström *et al.*, 1987; Björnsdóttir *et al.*, 2006). The IBH statuses of imported Icelandic horses and Icelandic horses born in Europe may not represent the exact same phenotype. The final Icelandic horse data, therefore, only included horses born in Europe.

6.4.2 Single SNP and multi-locus models

Schurink *et al.* (in press) published genomic regions associated with IBH in 188 Shetland pony mares using 50k SNP genotypes. In our study, several similar associated genomic regions within 1 Mb distance were identified in Shetland pony

Table 6.4 Windows explaining the largest percentages of insect bite hypersensitivity genetic variance in Shetland pony mares.

Top 20 associated windows[a]					SNP with highest model frequency within window				
ECA[b]	Position (Mb)[c]	Percentage of genetic variance explained[d]	Number of SNP	Percentage of iterations where variance explained >0.04%[e]	SNP name	SNP position (bp)	Model frequency[f]	Allele frequency[g] Cases	Controls
3	8	0.143	21	2.8	*BIEC2_810809*	8,098,240	0.29	0.67	0.54
3	17	0.141	30	3.1	*BIEC2_773375*	17,036,655	0.38	0.62	0.46
3	50	0.270	27	3.8	*BIEC2_779930*	50,444,836	0.68	0.45	0.29
3	51	0.161	25	3.2	*BIEC2_780595*	51,525,184	0.35	0.58	0.43
7	67	0.149	27	3.3	*BIEC2_1005528*	67,597,722	0.36	0.67	0.54
7	85	0.171	24	2.9	*BIEC2_1010550*	85,800,251	0.29	0.64	0.52
8	63	0.231	22	3.8	*BIEC2-1058160*	63,839,900	0.47	0.59	0.44
11	22	0.201	21	3.6	*BIEC2-143974*	22,769,190	0.32	0.50	0.35
11	23	0.149	24	3.1	*BIEC2_144465*	23,873,176	0.31	0.59	0.45
11	26	0.178	26	3.3	*BIEC2_145801*	26,946,633	0.20	0.51	0.39
11	32	0.193	31	4.1	*BIEC2_149137*	32,010,755	0.37	0.27	0.13
17	1	0.141	24	2.7	*BIEC2_366411*	1,024,001	0.27	0.45	0.31
17	6	0.147	28	2.6	*BIEC2_367597*	6,640,619	0.29	0.65	0.51
17	75	0.303	23	4.4	*BIEC2_384363*	75,401,514	0.67	0.67	0.52
17	76	0.159	18	2.3	*BIEC2_385267*	76,776,877	0.81	0.70	0.55
20	35	0.624	23	5.3	*UKUL3474*	35,643,200	2.03	0.56	0.37
20	41	0.176	21	2.9	*BIEC2_532511*	41,520,518	0.84	0.45	0.28
23	14	0.143	25	2.8	*TBIEC2_645769*	14,286,784	0.16	0.36	0.27
27	13	0.214	18	3.2	*BIEC2_705454*	13,198,799	0.78	0.71	0.54
28	41	0.154	24	3.2	*BIEC2_744415*	41,130,845	0.24	0.73	0.61

[a]top 20 1 Mb non-overlapping windows explaining the largest percentages of genetic variance; [b]*Equus caballus* autosome; [c]position of the window in Mb pairs, where for instance window position 8 Mb includes SNP located on that particular chromosome between 8 to 9 Mb; [d]percentage of total genetic variance explained by 1 Mb non-overlapping windows of consecutive SNP based on physical order (build EquCab2.0), averaged across post burn-in iterations, thereby including results from iterations that excluded the window from the model; [e]percentage of iterations (out of 1799 saved) during which the window captured over 0.04% of genomic variance (i.e. the expected percentage of variance explained by each window in an infinitesimal model); [f]percentage of iterations where SNP was modelled to have an effect; [g]frequency of the unfavourable allele

Table 6.5 Windows explaining the largest percentages of genetic variance for insect bite hypersensitivity in Icelandic horses.

Top 20 associated windows[a]					SNP with highest model frequency within window				
ECA[b]	Position (Mb)[c]	Percentage of genetic variance explained[d]	Number of SNP	Percentage of iterations where variance explained >0.04%[e]	SNP name	SNP position (bp)	Model frequency[f]	Allele frequency[g] Cases	Controls
1	7	0.215	25	4.5	*BIEC2_2768*	7,759,159	0.94	0.69	0.46
3	35	0.392	18	5.4	*BIEC2_776785*	35,897,049	0.45	0.46	0.26
4	24	0.161	17	2.7	*BIEC2_855840*	24,611,718	0.32	0.51	0.33
4	43	0.180	18	4.0	*BIEC2_861849*	43,590,939	0.51	0.69	0.49
5	26	0.176	20	3.2	*BIEC2_898729*	26,364,893	0.52	0.60	0.38
6	6	0.166	43	4.4	*BIEC2_937490*	6,127,639	0.69	0.54	0.30
7	55	0.179	24	3.8	*BIEC2_1001715*	55,888,542	0.36	0.35	0.18
9	78	0.182	30	3.9	*BIEC2_1106244*	78,254,394	0.44	0.43	0.25
11	40	0.266	21	3.5	*BIEC2_152809*	40,721,405	1.30	0.67	0.42
15	19	0.162	23	3.7	*BIEC2_293503*	19,944,954	0.66	0.55	0.34
15	20	0.211	22	3.1	*BIEC2_293623*	20,074,216	1.04	0.68	0.44
15	32	0.142	23	3.2	*BIEC2_301468*	32,220,117	0.31	0.59	0.41
15	33	0.381	32	4.9	*BIEC2-301721*	33,565,370	2.23	0.70	0.42
18	32	0.179	27	3.9	*BIEC2_431445*	32,561,292	0.69	0.54	0.34
19	15	0.186	24	3.5	*BIEC2_430270*	15,644,656	0.45	0.54	0.35
19	21	0.151	26	3.3	*BIEC2_431289*	21,754,514	0.33	0.75	0.57
20	30	0.162	17	2.2	*BIEC2_528135*	30,619,697	0.87	0.78	0.53
23	4	0.159	27	3.4	*BIEC2_637804*	4,466,955	0.49	0.74	0.55
X	59	0.658	29	7.9	*BIEC2_1126534*	59,703,839	1.04	0.58	0.33
X	60	0.282	29	3.6	*BIEC2_1126713*	60,238,370	1.32	0.59	0.33

[a]top 20 1 Mb non-overlapping windows explaining the largest percentages of genetic variance; [b]*Equus caballus* autosome; [c]position of the window in Mb pairs, where for instance window position 7 Mb includes SNP located on that particular chromosome between 7 to 8 Mb; [d]percentage of total genetic variance explained by 1 Mb non-overlapping windows of consecutive SNP based on physical order (build EquCab2.0), averaged across post burn-in iterations, thereby including results from iterations that excluded the window from the model; [e]percentage of iterations (out of 1799 saved) during which the window captured over 0.04% of genomic variance (i.e. the expected percentage of variance explained by each window in an infinitesimal model); [f]percentage of iterations where SNP was modelled to have an effect; [g]frequency of the unfavourable allele

mares on chromosomes 3, 11, 20 and 27. However, Schurink *et al.* (in press) used logistic regression fitting single SNP effects, while our Bayes-C method fitted all SNP simultaneously. Mucha *et al.* (2010) concluded that estimated variances of identified QTL were not overestimated when all SNP were fitted simultaneously, since the variance explained will be distributed across all SNP in high LD with the QTL and therefore cannot exceed the total variance (in contrast to single SNP analysis). Indeed, Sahana *et al.* (2010) compared various association mapping methods and showed that a Bayesian variable selection model that fitted all SNP simultaneously performed best overall. The Bayesian variable selection model using the posterior probability of a QTL in 1 cM overlapping regions to identify associated genomic regions had the highest power to map small QTL (i.e. explaining 2% of genetic variance) and most precise estimates of QTL locations. However, a mixed model analysis fitting random additive genetic effects and testing single SNP performed almost as well, although it was computationally more demanding and multiple testing correction was needed. Like in Sahana *et al.* (2010), analysis of the Shetland pony mare data using logistic regression with single SNP effects, as in Schurink *et al.* (in press), was computationally much more demanding than the Bayesian variable selection method used here and ignored dependencies between SNP. Although several similar associated genomic regions were identified using these two methods, Bayesian variable selection model using posterior probabilities of genomic regions is preferred as it is computationally less demanding, it does not require correction for multiple testing and it accounts for population stratification due to pedigree by fitting all SNP simultaneously.

6.4.3 Non-overlapping window approach

The window approach takes LD between SNP into account and is therefore a better criterion for QTL identification than posterior probabilities of single SNP (Sahana *et al.*, 2010; Onteru *et al.*, 2011). However, optimal choice of the size of a window is not clear, as a specific window may contain more than one QTL or a QTL may be spread over more than one window (Sun *et al.*, 2011). For example, after merging windows at 75 and 76 Mb on chromosome 17 in Shetland pony mares and performing another GWAS, the percentage of variance explained by this 2 Mb genomic region was 0.426, which roughly equals the sum of genetic variance explained by the two separate 1 Mb windows (Table 6.4). Because these 1 Mb windows were consecutive, the percentage of variance explained by the 2 Mb windows might be considered as total QTL variance (if indeed the two consecutive

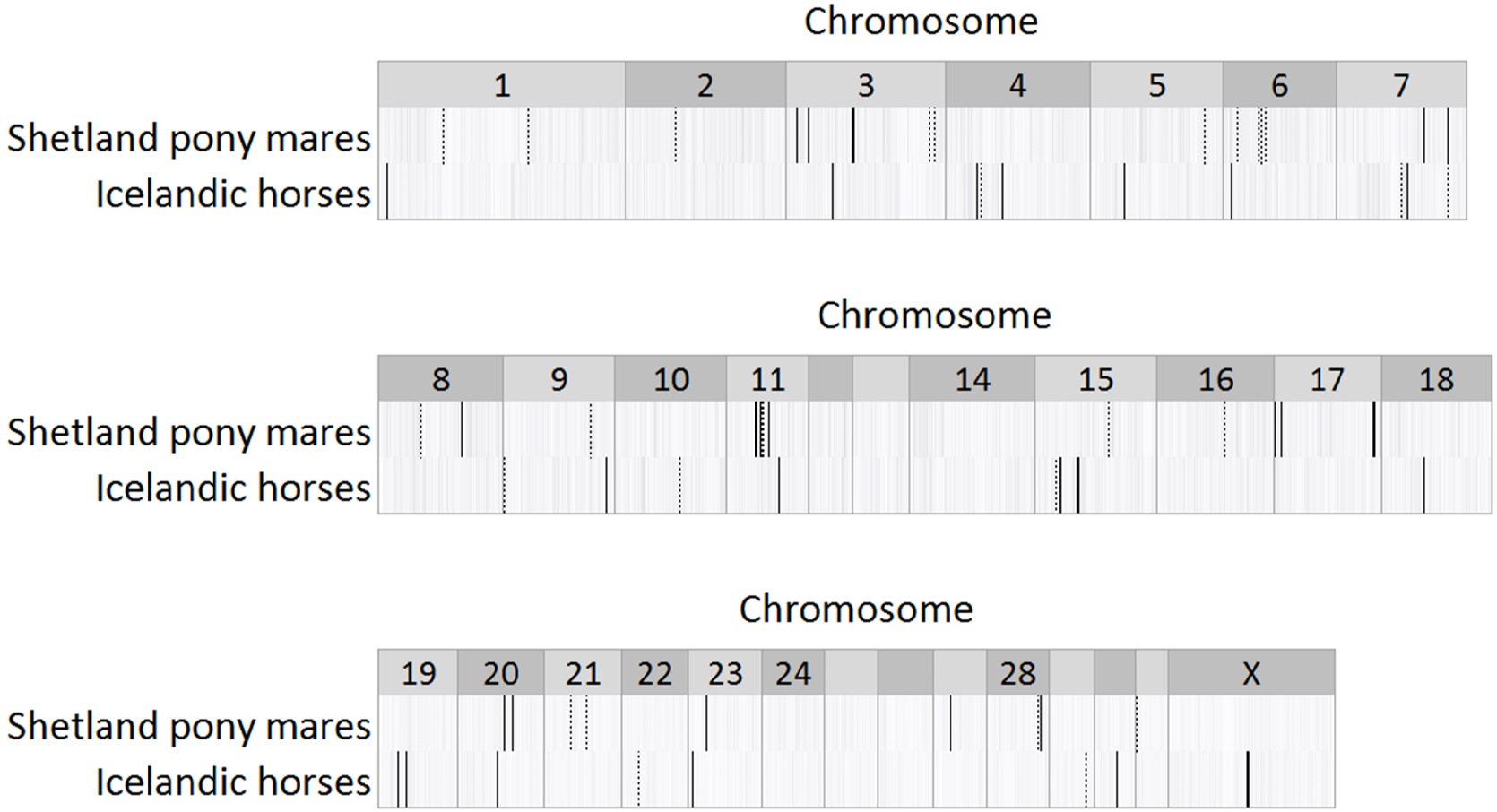

Figure 6.3 Heat map comparing the percentage of genetic variance of insect bite hypersensitivity explained by each window in Shetland pony mares and Icelandic horses born in Europe. Non-overlapping 1 Mb windows are based on the physical order of consecutive SNP across the genome (ECA1 to X; build EquCab2.0); black bars represent windows explaining ≥0.14% of genetic variance and dashed black bars represent windows explaining between 0.12 and 0.14% of genetic variance; diminishing grey colour represents a decrease in genetic variance (<0.12%) explained by windows.

1 Mb windows represent the same QTL), whereas the percentage of variance explained by each 1 Mb might each represent a proportion of QTL variance. However, the true QTL position might not be contained precisely in the window with strongest association. Precision of QTL mapping depends on several factors, such as the method of analysis, marker density, sample size and variance explained by the QTL (He *et al.*, 2010). In a simulated data set of binary phenotypes and SNP genotypes by Mucha *et al.* (2011), the mean distance of estimates from true QTL positions ranged from 0.30 to 0.77 Mb, depending on the method of analysis used. However, the SNP density simulated by Mucha *et al.* (2011) was higher than in our study. Because LD can differ between genomic regions (e.g. Smith *et al.*, 2005; Bohmanova *et al.*, 2010), LD within a genomic region could be used to determine the optimal size of a window in a given region, although further research is needed to determine the relationship between LD structure and optimal window size.

6.4.4 Genome-wide association study

Associated genomic regions identified in both breeds (Figure 6.3) suggest interesting candidate genomic regions to follow-up on. A simultaneous GWAS of both breeds is expected to increase power to detect associations, as more data would be included. However, GWAS across breeds will be less likely to detect SNP that are in LD with QTL in only one breed and will be more likely to detect SNP in LD with QTL across both breeds, provided LD phase is conserved across breeds (e.g. Dekkers and Hospital, 2002; De Roos *et al.*, 2008). To meet these requirements, SNP and QTL need to be physically close or, ideally, represent the actual mutation (which is unlikely). De Roos *et al.* (2008) concluded that roughly 50,000 SNP are required to have sufficient LD (i.e. ≥0.20) for genomic selection within a dairy cattle breed but that 300,000 SNP are required to find SNP that are in LD with the QTL across breeds. Persistency of LD phase extended less than 10 kb between bovine breeds that diverged hundreds of generations ago (De Roos *et al.*, 2008). The consistency of LD phase between Shetland ponies and Icelandic horses was not investigated. Shetland ponies and Icelandic horses did cluster together in the phylogenetic analysis of van de Goor *et al.* (2011), which used equine short tandem repeat loci. However, divergence of the breeds occurred many generations ago, thus LD from the ancestral population is expected to have been broken down (Hill and Robertson, 1968). Also, the current equine SNP density results in insufficient LD (roughly 0.3; Wade *et al.*, 2009) to expect to find SNP that are in LD with QTL across breeds.

6.4.5 Candidate genes

Research on IBH using the candidate gene approach or GWAS in horses has been limited. Using a candidate gene approach, Andersson *et al.* (2009) concluded that *SPINK5* (serine peptidase inhibitor, Kazal type 5) on ECA14 was not associated with IBH in Swedish-born Icelandic horses. In our study, no genomic regions associated with IBH were found on ECA14. Hořin *et al.* (2004) investigated polymorphisms in various immune response related genes to identify associations with *R. equi* and *L. intracellularis* that cause respectively lung and gastrointestinal infections in horses. Several polymorphisms were significantly associated with these infections, including microsatellite locus HMS01 on ECA15. Marti *et al.* (2005 in Chowdhary and Raudsepp, 2008) concluded that locus HMS01 is associated with IBH. In our study, genomic regions associated with IBH were identified on ECA15 but only in Icelandic horses. Various *IL1* (interleukin 1) related genes are located in or around these regions.

We anticipated a common genetic background of IBH across breeds, although breed-specific genetic influences on IBH cannot be excluded. However, SNP densities within genomic regions could differ between Shetland pony mares and Icelandic horses due to breed-specific edits based on MAF and call-rate. Also, LD between SNP and QTL might be present in one breed but absent in the other (e.g. De Roos, 2008), thereby impeding validation of QTL across breeds. Associated genomic regions identified in both Shetland pony mares and Icelandic horses were considered most interesting to follow-up on and were found on ECA3, 7, 11, 20 and 23 (Figure 6.3, Tables 6.4 and 6.5). However, positional candidate genes adjacent to associated genomic regions were identified only for the genomic region on ECA20. No other candidate gene with known function in immunology or allergy was identified in or adjacent to across-breed associated genomic regions. The equine lymphocyte antigen (ELA) class II region is located on ECA20 (spanning 32 and 33 Mb) between the associated genomic regions identified in the Shetland pony mares and Icelandic horses (Tables 6.4 and 6.5, Figure 6.3). ELA, or equine major histocompatibility complex, evokes an immune response by recognizing many foreign molecules (Bailey *et al.*, 2000). Both serological (Halldórsdóttir *et al.*, 1991; Marti *et al.*, 1992) and genomic research (Andersson *et al.*, 2012) have identified an association between ELA class II antigens and IBH. Andersson *et al.* (2012) concluded that the same allele at an ELA locus is associated with IBH in two distinct horse breeds and homozygosity across the ELA region increased IBH sensitivity. An association with IBH on ECA20 was also found by Schurink *et al.* (in press), although the identified region was approximately 8 Mb away from the ELA class II region. However, coverage within the region was poor for the Illumina® EquineSNP50

Genotyping BeadChip (Illumina Inc.) used by Schurink *et al.* (in press), but improved in the current equine HD chip. Associated genomic regions on ECA20 that were identified in the Shetland pony mares and Icelandic horses were within 2 Mb from the ELA class II region, which is reasonably close to confirm the impact of ELA class II region on IBH.

6.5 Conclusions and implications

The genome-wide association study performed here identified several genomic regions associated with IBH in both Shetland pony mares and Icelandic horses. On ECA20, associated genomic regions were identified in both breeds that were within 2 Mb from the equine lymphocyte antigen class II region containing candidate genes. Knowledge on genes associated with IBH will contribute to our understanding of its biology, enabling more efficient therapy, prevention and selection in order to decrease IBH prevalence. Sequencing candidate genes within the equine lymphocyte antigen class II region might identify the functional mutation. Selection on functional mutations, i.e. direct markers, is more effective than indirect markers (i.e. LD and linkage equilibrium markers) (Dekkers, 2004). However, genetic gain for marker-assisted selection using only a small number of significant markers to trace a limited number of QTL (although often with larger effects) is likely to be small because a large number of QTL are expected to explain genetic variation in complex traits (e.g. Hayes and Goddard, 2010). In genomic selection, dense genome-wide markers are used to estimate genomic breeding values based on marker effects across the entire genome. Marker density is assumed to be sufficient so that each QTL is in LD with at least one marker or with a set of markers. Therefore, genomic selection could potentially capture the total genetic variance for a complex trait (e.g. Hayes and Goddard, 2010). Possibilities for genomic selection on IBH in horse populations or even across horse populations and corresponding implications must be investigated before implementation is considered.

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7

General discussion

7.1 Introduction

The aim of the research described in this thesis was to increase our understanding of the genetic background of IBH to be able to reduce its prevalence in horse populations through selection and breeding. IBH is multifactorial in origin and therefore the basic animal breeding and genetic formula, P = G + E, is applicable. Here the phenotype (P) is the observed trait which is affected by underlying genetics (G) and various environmental factors (E). Regarding the phenotype, visual scoring of clinical symptoms (absent or present) is still the gold standard to determine IBH. Results from *in vivo* and *in vitro* tests may render a more refined phenotype (i.e. quantitative in nature). Various phenotypes of IBH and associated environmental factors are discussed in the first part of the general discussion, which ends with a preferred IBH data collection strategy. The second part of the general discussion focusses on the underlying genetics of IBH. Genetic variance of IBH is present (chapter 3 and 4) and several genomic regions were associated with IBH (chapter 5 and 6), thereby enabling selection to reduce prevalence of IBH. Potential genetic gain of several strategies based on own performance, progeny testing or genomic selection were simulated including a binary and quantitative phenotype. At the end of the general discussion implications for practical horse breeding against IBH are evaluated using results of simulated selection strategies.

7.2 Phenotype, environment and data collection

7.2.1 IBH phenotypes

IBH is often analyzed as a binary phenotype (i.e. affected or unaffected) in genetic and epidemiological research (chapter 2, 3 and 4; Broström *et al.*, 1987; Anderson *et al.*, 1988; Halldórdsóttir and Larsen, 1991; Eriksson *et al.*, 2008). IBH clinical symptoms are categorical in nature. However, an underlying non-observable scale is expected, indicated as the sensitivity to IBH. When this sensitivity is below a certain (unknown) threshold, a horse will be unaffected, while horses with a sensitivity above the threshold will show clinical symptoms. To approach this underlying sensitivity to IBH, severity of IBH clinical symptoms has been scored. However, heritability estimates based on severity of clinical symptoms hardly differed from heritability estimates of the binary phenotype (chapter 3; Eriksson *et al.*, 2008). Phenotypes of affected horses only (10 to 20% of a population) are subdivided, while phenotypes of unaffected horses (80 to 90% of a population) remain equal although their sensitivity to IBH can also vary greatly.

So far, diagnosis of IBH is based on a physical examination of clinical symptoms combined with the typical seasonality and recurrence of symptoms in subsequent years. Reduced itch after elimination of exposure to *Culicoides* spp. (e.g. stabling, protective rug) confirms the diagnosis, while other conditions causing itch like mite, louse or *Onchocerca cervicalis* infestation and dermatophytosis need to be ruled out (Pilsworth and Knottenbelt, 2004). Currently, results from *in vivo* and *in vitro* tests (henceforth referred to as diagnostic tests) contribute to the accuracy of IBH diagnosis, although the final diagnosis still relies on medical history of the horse and an evaluation of clinical symptoms (Baselgia *et al.*, 2006; Langner *et al.*, 2008; Sloet van Oldruitenborgh-Oosterbaan *et al.*, 2009). Results from diagnostic tests, which can be regarded as other but related phenotypes of IBH, include the release of specific allergic mediators (e.g. sulphidoleukotriene, histamine) (Riek, 1954; Kobelt, 2001; Baselgia *et al.*, 2006; Langner *et al.*, 2008; Wagner *et al.*, 2008), wheal size in intradermal testing (Riek, 1954; Braverman *et al.*, 1983; Fadok and Greiner, 1990; Langner *et al.*, 2008; Sloet van Oldruitenborgh-Oosterbaan *et al.*, 2009) and *Culicoides* spp. specific IgE or IgG determined by ELISA (Frey *et al.*, 2008; Langner *et al.*, 2008). Diagnostic tests often focus on IgE-mediated mast cell degranulation thereby potentially ignoring other involved (unknown) immunological mechanisms (Marti *et al.*, 2008), while IBH clinical symptoms are a result of and encompass all mechanisms. To test a large number of samples using a diagnostic test has practical drawbacks. For instance, Marti *et al.* (1999) showed that fresh blood samples are needed as sulphidoleukotriene release is already impaired after 24h storage. Also, compared to scoring IBH clinical symptoms, diagnostic testing needs invasive procedures (often only allowed to be performed by veterinarians) and therefore requires ethical considerations. On the other hand, diagnostic test results might better differentiate horses with regard to their sensitivity to IBH, in contrast to IBH scored as a binary phenotype, and might depend less on exposure or presence of *Culicoides* spp. Specific management of these sensitive horses might even prevent the development of clinical symptoms. Moreover, sensitization to *Culicoides* spp. allergens as detected by various diagnostic tests precedes IBH clinical symptoms (Wagner *et al.*, 2008) and can therefore be detected at a younger age (Wagner *et al.*, 2006; Marti *et al.*, 2009). An increased differentiation will better capture the genetic variance and contribute to a higher rate of genetic gain (i.e. decrease in sensitivity to IBH and thus a reduced prevalence).

7.2.2 Environmental factors

Various environmental factors are associated with IBH prevalence (chapter 2, 3 and 4) and can be divided into factors related to the horse (e.g. age, body condition),

factors related to *Culicoides* spp. activity and density (e.g. region, climate) and management related factors (e.g. stabling, use of protective rug). Knowledge of environmental factors associated with IBH will contribute to a more accurate phenotype and thereby more accurate breeding values.

Horse related factors

Horse related factors associated with IBH prevalence were age, coat colour and withers height category (chapter 2, 3 and 4). Sex of the horse was associated with IBH prevalence in some studies (Braverman *et al.*, 1983; Broström *et al.*, 1987; Eriksson *et al.*, 2008), but could not be tested as potential risk factor in our research as only mares were included. In general, contradicting results concerning horse related factors associated with IBH are reported in literature. For example, prevalence in mares was significantly lower compared to stallions and geldings in the study of Braverman *et al.* (1983) and Eriksson *et al.* (2008), whereas stallions were least affected in the study of Broström *et al.* (1987). Many other studies failed to find a significant difference in prevalence between sexes (Anderson *et al.*, 1988; Halldórdsóttir and Larsen, 1991; Steinman *et al.*, 2003; Björnsdóttir *et al.*, 2006; Peeters *et al.*, 2010). It remains questionable whether IBH associated horse related factors like sex are physiological in nature [e.g. hormonal differences and perspiration composition of sexes (Broström *et al.*, 1987)] or are indirectly related to *Culicoides* spp. exposure [e.g. management differences between sexes like stabling (Eriksson *et al.*, 2008)].

Body condition (i.e. the amount of stored fat in a body) was scored simultaneously with IBH clinical symptoms in Shetland pony mares only and had a nearly significant effect on IBH prevalence (chapter 2). Mares with either low or high body condition score showed increased odds to be diagnosed with clinical symptoms compared to mares with a normal body condition score. To minimize IBH clinical symptoms in horses, it therefore seems wise to maintain a normal body condition. Our results are comparable to results from Peeters *et al.* (2010) who found that IBH prevalence was increased in underweight (25.0%), overweight (14.6%) and severely overweight Belgian warmblood horses (13.8%) compared to prevalence in slightly overweight horses (9.4%) and horses with normal body condition (8.8%). In humans, obesity is considered a risk factor for allergies (e.g. Irei *et al.*, 2005; Kwon *et al.*, 2006; Coogan *et al.*, 2009) and underweight was associated with having asthma as well (Kwon *et al.*, 2006). Hersoug and Linneberg (2007) hypothesized that immunological changes at least partly contribute to the increased risk of allergies in obese individuals as white adipose tissue secretes more adipokines and cytokines thereby resulting in a decreased immunological tolerance to antigens and skewing towards a Th2-biased

immune response. Inflammation is suggested to be the basis for the relation between obesity and allergic diseases like asthma (e.g. Canöz *et al.*, 2008; Michelson *et al.*, 2009). Indeed, specific inflammatory cytokines like TNF-α, IL-6 and leptin were significantly increased in obese affected individuals compared to unaffected and non-obese affected individuals (Canöz *et al.*, 2008). Like in humans, an increased production of inflammatory cytokines was observed in obese horses (Vick *et al.*, 2007; Adams *et al.*, 2009). Also, the production of cytokines differed between IBH affected and unaffected horses (Hamza *et al.*, 2007; Cunningham and Dunkel, 2008). Moreover, a higher genetic predisposition for overweight was associated with a higher genetic sensitivity to IBH (Schurink *et al.*, 2010) which indicates that both traits seem to be influenced by common genes. Likewise, Thomsen *et al.* (2007) found a significant genetic correlation between asthma and obesity in humans and Hallstrand *et al.* (2005) showed that a considerable part of the covariance between asthma and obesity is caused by shared genes. Selection in one trait will therefore result in a correlated response in the other trait.

Complex maturation and education of immune responses during early postnatal life are suggested to highly influence the expression of allergies in adulthood. Both prenatal and early postnatal environmental exposure to allergens presumably affects susceptibility to allergies in humans (Holt and Jones, 2000; Halken, 2003; Holgate and Polosa, 2008). In horses, transfer of maternal IgE including *Culicoides* spp. specific IgE from affected dams to foals does occur (Wagner *et al.*, 2006; Marti *et al.*, 2009). Endogenous IgE synthesis in foals started between 6 and 11 months of age when maternal IgE had diminished (Wagner *et al.*, 2006; Marti *et al.*, 2009). Results of Marti *et al.* (2009) did not show that foals' IgE response was maternally primed. Sommer-Locher *et al.* (2012) found that prevalence of IBH among foals imported from Iceland was equal to prevalence in locally (Europe) born foals and concluded that allergen exposure during the first 10 months of life was irrelevant for the development of IBH. Prevalence in foals imported from Iceland was 6.0% and significantly lower than prevalence in horses that were imported at an older age (46.3%). Broström *et al.* (1987) and Halldórdsóttir and Larsen (1991) found a significantly higher IBH prevalence among Icelandic horses imported from Iceland (26.2 and 26.9% respectively) compared to prevalence among locally born Icelandic horses (6.7 and 8.2% respectively). Data included exclusively adult horses, as average age at import was 6 years (Halldórdsóttir and Larsen, 1991). Data on age at import is needed to determine whether an Icelandic horse imported from Iceland has an increased risk to develop IBH clinical symptoms. In our study (chapter 6), age at import was unknown for Icelandic horses imported from Iceland. Moreover, IBH status of these imported horses and Icelandic horses born in Europe might not

represent the exact same phenotype (as discussed in chapter 6). Excluding Icelandic horses imported from Iceland from the genome-wide association study (chapter 6) therefore is justified. A maternal effect contributed to IBH variance as established by genetic analysis, although it did not improve the fit of the animal model based on the natural logarithm of Bayes Factor (chapter 4). A maternal effect is however not necessarily restricted to early postnatal environment and might encompass more aspects like management by the owner besides maternal transfer of IgE. More knowledge of the maturation and education of the immune system of young horses and environmental factors associated with these processes is required to establish a potential effect on the development of IBH clinical symptoms.

Culicoides *spp. and management related factors*

When *Culicoides* spp. are absent, no IBH clinical symptoms are observed. Variations in *Culicoides* spp. population density between and within years were related to changes in microclimate throughout the year (Takken *et al.*, 2008). Van der Rijt *et al.* (2008) showed that the number of *Culicoides* spp. caught was greatest during sunset. Wind and rain impaired the collection of *Culicoides* spp. (van der Rijt *et al.*, 2008; Meiswinkel *et al.*, 2000), while temperature was positively and humidity negatively related to number of *Culicoides* spp. caught (Meiswinkel *et al.*, 2000). IBH prevalence was increased in regions with low rainfall, few cold days and many warm days (van Grevenhof *et al.*, 2007). Many studies identified an association between region or habitat and IBH prevalence (Broström *et al.*, 1987; Eriksson *et al.*, 2008; van den Boom *et al.*, 2008). Factors that determine *Culicoides* spp. activity and density highly contributed to differences in IBH prevalence between regions, years and even months and were consistent throughout multi-disciplinary studies.

Various preventive measures aim at reducing exposure to *Culicoides* spp. Stables need to be suitable in order to keep out *Culicoides* spp. (Meiswinkel *et al.*, 2000) and sensitive horses need to be stabled during sunset, when *Culicoides* spp. are active (van der Rijt *et al.*, 2008). Many insecticides are available, but only a few have been tested scientifically and varied in efficacy (de Raat *et al.*, 2008; Papadopoulos *et al.*, 2010). Protective rugs are often used to reduce exposure to *Culicoides* spp. in IBH sensitive horses, but use of rugs encounters drawbacks (e.g. during warm weather) and is considered undesired. Also, sensitive horses could be moved to low-risk regions. Reduced exposure to *Culicoides* spp. has the potential to reduce IBH prevalence, although strict management (e.g. timely stabling every day) of owners is needed to succeed and several drawbacks are encountered when applying these measures. Knowledge of macro- and especially microenvironment

(e.g. breeding sites, lee at pasture, habitat) as well as applied preventive and therapeutic measures has the potential to increase reliability of IBH phenotypes (i.e. clinical symptoms), as presence of symptoms highly depends on exposure to *Culicoides* spp.

Environmental factors and diagnostic test results

Environmental factors might affect diagnostic test outcomes. For instance, Baselgia *et al.* (2006) found a significantly higher sulphidoleukotriene release in IBH affected geldings compared to affected mares. The proportion of negative test results in affected horses was significantly higher approximately half a year after last exposure to *Culicoides* spp. compared to the proportion of negative test results in affected horses during or just after exposure. However, effects of environmental factors on diagnostic test results often are not eliminated, as sample size in diagnostic studies are small. Before a widespread application of a diagnostic test can be considered in practice as well as in genetic evaluations, it is needed to investigate potential associations between environmental factors (e.g. sex, age and breed of the horse, use of preventive measures, month of testing) and test results in a large sample to improve its reliability and accuracy.

7.2.3 Data collection

Observations on IBH clinical symptoms that were used in our research (chapter 2, 3 and 4) were gathered by inspectors during obligatory foal inspections. Foals were inspected for studbook registration while their dam needed to be present and was scored for IBH. Inspectors scored mares on a three point scale: 0 = no clinical symptoms, 1 = dubious or mild clinical symptoms and 2 = clear clinical symptoms. Part of the inspectors participated in a training session at the start of data collection where inspectors were instructed to score clinical symptoms by an experienced veterinarian. As a consequence of this data collection method, all mares with foals were scored, thereby representing an unselected large sample of the active female breeding population. No phenotypes on males were collected. However, their phenotypes are considered less reliable as males (especially breeding stallions) are more often kept stabled and therefore are potentially less exposed to *Culicoides* spp. In Shetland pony mares, inspector, region and family were partly confounded as discussed in chapter 2 and 3. To improve the quality of the data, it is advised that inspectors score in different regions to prevent confounding with region and family, as Shetland pony breeding is regionally organized. Care should be taken when collecting data to prevent confounding between environmental and/or genetic factors to enable accurate estimation of

environmental effects in epidemiological research but also prevent bias in genetic evaluations.

In general, repeatability of IBH over years was lower than expected (chapter 2, 3 and 4) and indicated that repeated observations on IBH clinical symptoms will improve accuracy of estimated breeding values. Observed IBH clinical symptoms scored by inspectors are cross-sectional in nature and neither include medical history concerning IBH (typical seasonality and recurrence of symptoms) nor data on applied preventive or therapeutic measures besides stabling. Although owners can provide information concerning medical history and applied measures through a questionnaire, response rate will be far from 100% and owners of affected horses are more likely to respond (e.g. van den Boom *et al.*, 2008). Questionnaires are, therefore, not a good way to collect data for a representative sample of the population. Data collection by inspectors is easily implemented in routine studbook inspections and allows for the collection of an unselected large sample of the active female breeding population and the identification of IBH sensitive families (chapter 3 and 4).

Scoring severity of clinical symptoms did not improve the genetic differentiation of affected horses. However, IBH clinical symptoms are fully conditional on exposure to *Culicoides* spp. Unexposed horses are obviously scored as unaffected, despite a potential sensitivity to IBH. To ensure reasonable exposure to *Culicoides* spp., it seems wise to score not too early within the *Culicoides* spp. 'season'. A binary phenotype is sufficient to estimate breeding values, although this does not mean that scoring IBH into three or even more categories is redundant. Dubious and mild clinical symptoms (score 1) should be separated to improve their differentiation. Analyses will determine if and which categories could be merged. A genome-wide association study asks for a reliable individual IBH phenotype, as incorrect classification of horses will obscure the association between phenotype and genotype. A quantitative phenotype has the potential to improve individual reliability of a phenotype (as discussed in sections 7.2.1 and 7.2.2) and seems therefore more suitable to perform such a genome-wide association study. In our research, in absence of a quantitative phenotype, reliability of IBH binary phenotypes in the genome-wide association studies (chapter 5 and 6) increased because an experienced veterinarian confirmed and scored the observed clinical symptoms and an IBH-related questionnaire was conducted.

Data on *Culicoides* spp. and management related factors like region within the country, applied preventive and/or therapeutic measures and microenvironment has the potential to increase reliability of IBH phenotypes and therefore accuracy of breeding values, as these factors are highly associated with exposure to

Culicoides spp. and thereby IBH clinical symptoms. However, catching *Culicoides* spp. using for instance sticky insect traps enables a direct assessment of *Culicoides* spp. density while data on *Culicoides* spp. related factors only yield an indirect assessment of density. Horse related factors like age and sex can often be retrieved from studbooks, while it remains questionable whether their effects are physiological in nature as various studies suggest them to be related to management decisions and *Culicoides* spp. exposure.

To conclude, specific requirements on data collection should be met to increase accuracy of breeding value estimation and selection decisions based on IBH clinical symptoms. Horses should be scored more than once, as repeatability was limited, but not too early in the *Culicoides* spp. 'season' to ensure sufficient exposure. Inspectors should ask owners for the medical history of their horse and recurrence of IBH clinical symptoms. Local microenvironment and applied preventive or therapeutic measures should be recorded, although direct assessment of *Culicoides* spp. density is preferred. The required phenotype depends on the intended goal (e.g. breeding value estimation, genome-wide association study), although more research is needed to prove the applicability of a quantitative phenotype (e.g. diagnostic test results) in breeding against IBH.

7.3 Genotype

Our research on Shetland pony and Friesian horse mares (chapter 3 and 4) revealed genetic variance in sensitivity to IBH. Proportion of genetic variance explained by all genetic markers (chapter 6) in Shetland pony mares (13%) and Icelandic horses (28%) were similar to heritability estimates in these breeds (chapter 3). Research on IBH observations in 1,250 Swedish-born Icelandic horses rendered nearly equal IBH heritability estimates (Eriksson *et al.*, 2008). Van den Boom *et al.* (2008) found that IBH prevalence was significantly higher in horses that were used for breeding compared to horses that were used in competition or that had no defined use. Rate of genetic gain per generation (i.e. reduction in IBH prevalence) of various selection strategies were simulated. Results and implications are discussed in the following sections.

7.3.1 Selection strategies

The rate of genetic gain per generation, defined as the reduction in IBH prevalence, was simulated for several selection strategies using Sel*A*ction software (Rutten *et al.*, 2002). Sel*A*ction software (Rutten *et al.*, 2002) incorporates reduction of genetic variance due to selection, that is the Bulmer effect, and uses pedigree

Table 7.1 Input parameters of simulated selection strategies based on EBV for a binary or quantitative phenotype.

Parent	Number	Progeny per parent	Selected proportion
Sires	1,250 (300)	10 or 20 (50)	1, 0.5 or 0.1[a]
Dams	15,000	1♂ and 1♀	1 or 0.925
Phenotype	Definition	Heritability	Phenotypic variance
Binary[b]	With or without symptoms	0.24	1.0
Quantitative	*C. obsoletus* IgE levels	0.24	0.2[c]
Strategy	Generation interval	Selection	
Own performance	6 years	Single-stage	
Progeny testing	10 years	Binary: single-stage, quantitative: two-stage	
Genomic selection	4 years	Single-stage	

[a]Sire EBV did not include own performance for clinical symptoms (i.e. binary phenotype) in any of the strategies. All but one strategy were based on single-stage selection; the progeny testing strategy for the quantitative phenotype included two-stage selection. Total selected proportion remained 0.5 (or 0.1); 80% (or 50%) of the sires were selected on EBV that included own performance and subsequently 62.5% (or 20%) of the sires were selected on EBV that included progeny performance.
[b]Simulated parameters for the binary phenotype were based on the underlying unobserved sensitivity to IBH.
[c]Phenotypic variance of the quantitative phenotype was based on variance of *C. obsoletus* specific IgE levels in serum samples of roughly 50 affected and 50 unaffected Shetland pony mares.

information to predict the accuracies of breeding value estimates based on best linear unbiased prediction (BLUP) context. A breeding value is the genetic value of an individual that is passed on to its progeny. Individuals with the best breeding values are selected to produce the next generation. Sel*A*ction software (Rutten *et al.*, 2002) offers the opportunity to determine the impact of using different information sources like own performance, performance of relatives (e.g. sibs, progeny) and genomic data on the accuracy of breeding values and expected rate of genetic gain. Simulated selection strategies were based on own performance, progeny testing and genomic data. Input parameters of simulated strategies concerning a binary and quantitative phenotype are presented in Table 7.1. The binary phenotype is henceforth referred to as “clinical symptoms” and the quantitative phenotype as “diagnostic test”.

A population with discrete generations was simulated that represents the Shetland pony breeding population (Table 7.1). A simplified breeding program with selection for IBH phenotype only was considered. All selection decision were based on estimated breeding values (EBV) that included own performance, progeny

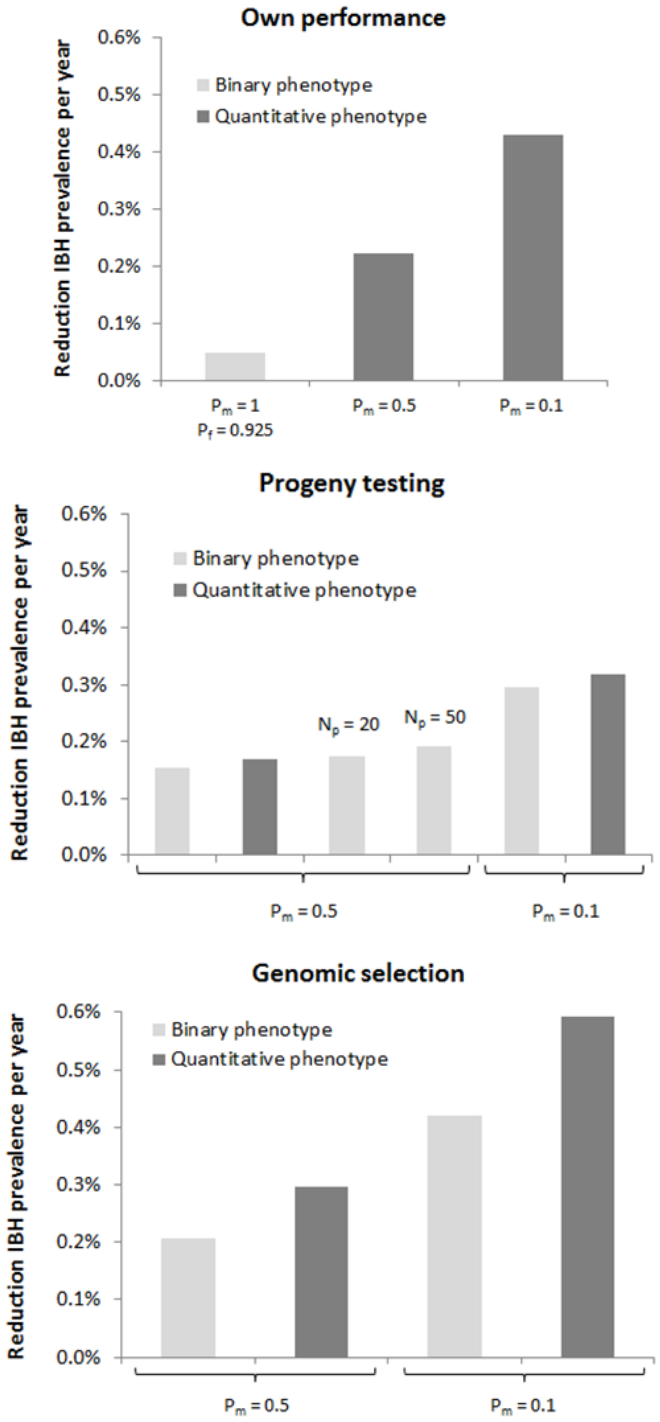

Figure 7.1 Expected genetic gain per year in simulated strategies based on EBV that included own performance, progeny performance or genomic data. P_m and P_f are the selected proportions in respectively sires and dams. N_p is the number of tested progeny per sire; N_p = 10 if no number is not mentioned. All but one strategy were based on single-stage selection. Two-stage selection was performed in progeny testing strategies for the quantitative phenotype (i.e. diagnostic test): selection was based on EBV that included their own performance, followed by selection based on EBV that included progeny performance as well. Simulated percentage of genetic variance explained by genetic markers was 13% for a binary phenotype (i.e. IBH clinical symptoms) and 28% for a quantitative phenotype.

performance, genomic data or combinations of these information sources. Subsequent reference to “own performance”, “progeny testing” and “genomic selection” means that selection decisions were based on EBV that included own performance, progeny performance and genomic data respectively. All but one strategy were based on single-stage selection (Table 7.1). Two-stage selection was performed in progeny testing strategies for the diagnostic test only; sires were selected on own performance, followed by selection based on progeny testing. An additional simulated progeny testing strategy included 300 sires with 50 offspring (paternal half-sibs) each to represent studbooks like the Friesian horse population.
Selected proportion in sires was 1, 0.5 or 0.1 (Table 7.1). Concerning IBH clinical symptoms, EBV of sires did not include own performance in any of the strategies as sires are more often kept stabled and are therefore potentially less exposed to *Culicoides* spp. Selection in dams was based on their own performance. Selected proportion in dams equaled 0.925 (= 1 – prevalence).
Generation interval was simulated to be 6 years in selection strategies based on own performance, 10 years in progeny testing strategies and 4 years in genomic selection (Table 7.1). Expected rate of genetic gain of simulated selection strategies per generation were divided by generation interval to enable comparison of strategies with different generation interval. Therefore, genetic gain per year (defined as reduction in IBH prevalence per year) were presented (Figure 7.1).

Selection including own performance

IBH prevalence reduced with 0.05% per year (Figure 7.1) in a strategy where dams were selected (P_f = 0.925) on own performance for clinical symptoms (i.e. EBV that included own performance). As own performance for IBH clinical symptoms highly affects a dam’s EBV, dams excluded from breeding most likely showed clinical symptoms (excluded proportion therefore equaled the prevalence). EBV of sires did not include own performance for IBH clinical symptoms. Accuracy of EBV was therefore low and proportion of selected sires was set to 1.
For the diagnostic test, prevalence reduced with 0.22% per year when half of the sires were excluded from breeding (P_m = 0.5) based on their own performance (Figure 7.1). Genetic gain almost doubled when selected proportion in sires (= P_m) lowered from 0.5 to 0.1 (Figure 7.1). Selection in dams (P_f = 0.925) on own performance only slightly increased genetic gain per year (data not shown).

Selection including progeny testing

A sire's EBV for clinical symptoms excluded their own performance but included progeny (paternal half-sibs) performance. Prevalence reduced with 0.15% per year when half of the sires were excluded (P_m = 0.5) and number of tested progeny was 10. Genetic gain increased with 12% when 20 instead of 10 progeny were tested and with another 11% when 50 instead of 20 progeny were tested (Figure 7.1). Genetic gain doubled when selected proportion in sires decreased from 0.5 to 0.1 and number of tested progeny was 10 (Figure 7.1). Simulated single-stage progeny testing strategies for IBH clinical symptoms outperformed selection based on own performance of dams only (Figure 7.1).

A sire's EBV for the diagnostic test included both own performance and progeny performance. Simulated progeny testing strategies for the diagnostic test were therefore based on two-stage selection; the first selection decision was based on a sire's own performance only, whereas the second decision was based on progeny testing. Using the diagnostic test, prevalence reduced with 0.17% per year when half of the sires were selected (P_m = 0.5) and number of tested progeny was 10 (Figure 7.1). Genetic gain increased with 8% when 20 instead of 10 progeny were tested and with another 4% when 50 instead of 20 progeny were tested (data not shown). Genetic gain increased 87% when selected proportion in sires (with 10 tested progeny) decreased from 0.5 to 0.1 (Figure 7.1). Simulated two-stage progeny testing strategies for the diagnostic test did not outperform selection on own performance only (Figure 7.1). Selection in dams (P_f = 0.925) based on their own performance, only slightly increased genetic gain per year for both phenotypes (data not shown).

Genomic selection

We assumed that genetic markers explained 13% of the genetic variance for IBH clinical symptoms in the Shetland pony mare population (based on results in chapter 6). Genetic gain that would result from using genomic breeding values was calculated. Prevalence reduced with 0.21% per year when selected proportion in sires was 0.5 (Figure 7.1). Genetic gain doubled when selected proportion in sires decreased from 0.5 to 0.1 (Figure 7.1). Genomic selection outperformed selection on own performance or progeny testing.

Genomic selection included selection on genomic breeding values only. Potential own performance of sires for the diagnostic test was ignored. We assumed that genetic markers explained 28% of the genetic variance for the diagnostic test. Prevalence reduced with 0.30% per year when selected proportion in sires was 0.5

(Figure 7.1). Again, genetic gain doubled when selected proportion in sires decreased from 0.5 to 0.1 (Figure 7.1). Genetic gain increased 25% when EBV also included a sire's own performance for the diagnostic (data not shown), assuming no effect on generation interval (remained 4 years). Again, genomic selection outperformed selection on own performance and/or progeny testing (Figure 7.1).

Implications of selection on clinical symptoms

Exposure to *Culicoides* spp. is crucial for collection of phenotypes on IBH clinical symptoms, as no symptoms will be observed without exposure. Unexposed horses therefore do not provide any information for selection decisions. Moreover, genetic gain resulting from selection on IBH clinical symptoms (i.e. EBV that included own performance) will be limited. Horses without clinical symptoms are difficult to discriminate although their (unobserved) sensitivity to IBH varies substantially. Indeed, prevalence reduced 0.05% per year (= 0.3% per generation) in a strategy where dams were selected (P_f = 0.925) on their own performance for clinical symptoms (Figure 7.1). As own performance for IBH clinical symptoms highly affects an individual's EBV, individuals excluded from breeding most likely showed clinical symptoms. The selection response largely depends on IBH prevalence. Genetic gain will decrease over generations as prevalence decreases due to breeding against IBH. Due to dependency on exposure to *Culicoides* spp. and prevalence within a population, excluding all horses with IBH clinical symptoms from breeding (actually, with undesired EBV) might not be the most effective way to substantially reduce prevalence within a population. A diagnostic test (as discussed in section 7.2.1) offers opportunities to improve genetic gain. Implications for breeding against IBH using a diagnostic test are discussed in the next section.

Prevalence in progeny of affected dams was increased (chapter 3 and 4; Eriksson *et al.*, 2008), although still a large proportion of progeny of affected dams (>0.8) did not exhibit IBH clinical symptoms. Accuracy of EBV is higher when EBV incorporate information sources like family and progeny information besides own performance. Exposure to *Culicoides* spp. is essential within families and progeny groups but not necessary in all family members in strategies involving progeny testing for IBH clinical symptoms as opposed to selection on own performance only. However, as discussed in section 7.2.3, environmental factors indirectly related to *Culicoides* spp. exposure should be collected or, preferably, a direct assessment of *Culicoides* spp. density should be made. Progeny testing is needed to achieve reasonable genetic gain (3.0% per generation where P_m = 0.1 and N_p = 10) for IBH clinical

symptoms, although expected reduction in prevalence highly depends on the selected proportion in sires and to a lesser extent on the number of progeny tested (Figure 7.1).

Implications of selection on a diagnostic test

Selection on own performance only could be more efficient and applied to sires as well when based on a diagnostic test rendering phenotypes like *C. obsoletus* specific IgE levels (Figure 7.1). Features of such a diagnostic test will determine whether selection can be performed independent of exposure to *Culicoides* spp. and at a younger age (compared to selection on clinical symptoms, as discussed in section 7.2.1) thereby increasing expected genetic gain per year. Testing 20 or more progeny per sire only slightly increased genetic gain compared to testing 10 progeny (data not shown). Rate of genetic gain per generation (3.2%) in progeny testing strategies outperformed rate of genetic gain per generation (2.6%) in selection on own performance only. However, genetic gain per year in progeny testing strategies was lower than genetic gain per year when selection was on own performance only (Figure 7.1). Progeny testing strategies increased generation interval to roughly 10 years as a sufficient number of progeny need to be old enough to be able to be tested, whereas selection on own performance is established around or even before the age of 6 years. Moreover, overall proportion of sires selected was assumed to be equal for both single-stage and two-stage strategies (Table 7.1); at the end an equal number of sires would be approved for breeding. Proportion of sires selected on own performance only therefore differed between single- (0.5 or 0.1) and two-stage selection strategies (0.8 or 0.5). As breeding values based on own performance become more accurate (and thereby increase genetic gain) when a heritability is higher, it becomes less necessary to test progeny (and thereby increase generation interval).

Implications for future breeding

The simulated selection strategies focused on IBH only. However, the breeding goal within a horse population includes more traits such as performance, conformation, movement and health. Obtained reduction in IBH prevalence therefore represents an upper limit of what can be achieved through selection. Genetic correlations between IBH and breeding goal traits might hamper or facilitate genetic gain. A positive genetic correlation between IBH and a breeding goal trait could improve reduction in IBH prevalence, especially when the index trait is easily and reliably scored. However, no significant genetic correlations between IBH and breeding goal traits (performance, conformation, movement) were found in a preliminary

analysis of EBV in Shetland pony and Friesian horse sires (data not shown). This suggests that current selection, in which IBH is not included in the breeding goal, does not affect IBH prevalence. Furthermore, it suggests that information on IBH phenotypes is essential for generating genetic response in IBH prevalence through selection.

Selection in dams yields additional genetic gain, although its benefit is limited (data not shown). However, studbooks can only guide selection in dams through for instance positive discrimination during inspections and voluntary performance tests, whereas owners fully determine whether their dam is suitable for breeding purposes.

Selection against IBH could be based on EBV that include own performance only when a suitable diagnostic test that measures sensitivity to IBH in a quantitative manner (with moderate heritability) becomes available. A potential decrease in generation interval (as sensitization to *Culicoides* spp. allergens as detected by various tests precedes clinical symptoms) and especially a possible independency of exposure to *Culicoides* spp. as compared to selection on clinical symptoms (discussed in section 7.2.1) should be highly valued. However, a random sample of affected and unaffected individuals from both high- and low-risk regions should be tested with a diagnostic test, to prove its applicability in breeding against IBH. At present, in the absence of such a suitable diagnostic test, selection aimed at reducing IBH prevalence should be based on testing 10 but preferably more progeny for IBH clinical symptoms. Reliability of clinical symptoms and therefore genetic gain could increase when specific requirements on data collection, as discussed in section 7.2.3, are met. Moreover, selection in sires should be strict to accomplish a reasonable reduction in IBH prevalence. Yet, genomic selection against IBH has the potential to outperform selection on own performance and progeny testing for both IBH clinical symptoms and a diagnostic test (Figure 7.1). Implications concerning genomic selection against IBH in horse populations are discussed in section 7.3.2.

7.3.2 Genomic selection

Simulations of various selection strategies showed that genetic gain per year using genomic selection was superior to genetic gain per year on own performance or progeny testing (Figure 7.1). Genomic selection uses all genome-wide markers, thereby capturing total genetic variance, to select the best individuals (Meuwissen *et al.*, 2001). Marker effects are estimated in a reference population that has both phenotypes and genotypes to predict genomic breeding values (sum of effects across all markers). Subsequently, genomic breeding values can be estimated in

individuals that have genotypes but lack phenotypes (e.g. in selection candidates). Marker effects have to be updated frequently to maintain a reasonable accuracy of genomic breeding values due to decay of LD between genetic markers and QTL across generations (Sonesson and Meuwissen, 2009). Genomic breeding values can therefore be estimated at a young age (before clinical symptoms develop) and independent of exposure to *Culicoides* spp. Genomic selection has the potential to shorten generation interval as opposed to progeny testing. Effects of shortening generation interval were incorporated in simulated selection strategies (Table 7.1) as genetic gain per year was calculated (Figure 7.1) to enable comparison of strategies with different generation interval. However, rate of genetic gain per generation in a progeny testing strategy (P_m = 0.1 and N_p = 10) for both IBH clinical symptoms and a diagnostic was higher (3.0 and 3.2% respectively) than rate of genetic gain per generation in genomic selection (1.7 and 2.4% respectively) or selection on own performance for the diagnostic test (2.6%). Assumed generation interval (Table 7.1) therefore highly influenced expected genetic gain per year. A higher heritability and thereby more reliable phenotype increased accuracies of genomic breeding values (e.g. de Roos *et al.*, 2009). Indeed, accuracy of genomic breeding values and thereby genetic gain was greater in a diagnostic test than in IBH clinical symptoms (Figure 7.1), as percentage of genetic variance explained by genetic markers for the diagnostic test (28%) was assumed to be greater than the percentage of genetic variance explained by genetic markers for IBH clinical symptoms (13%).

Several genomic regions were associated with IBH in Shetland pony mares and Icelandic horses using some tens of thousands of genetic markers (chapter 5 and 6). Breed-specific genomic regions associated with IBH were identified, whereas across breed associations were somewhat limited (chapter 6). Size of the collected Shetland pony mare and Icelandic horse population is considered to be too small to be able to implement breed-specific genomic selection against IBH, as size of the reference population determines accuracy of genomic breeding values (Goddard, 2009; VanRaden *et al.*, 2009; Daetwyler *et al.*, 2010). Calus (2010) discussed that at least 1,000 dairy cattle bulls with accurate phenotypes (e.g. conventional breeding values) were needed in the reference population to obtain accuracies of genomic breeding values in individuals without phenotypes that were higher than accuracies of breeding values based on pedigree indexes. As many horse populations are small, it is questionable whether breed-specific reference populations could be obtained including sufficient sires with reliable phenotypes. In addition, the number of available markers is considered too few to ensure LD between markers

and QTL and similar LD phase across breed (e.g. de Roos *et al.*, 2008) to perform genomic selection across horse populations. Design of a reference population affects accuracy of genomic breeding values as well (e.g. Pszczola *et al.*, 2012). A wide range of genotypes and phenotypes should be represented in the reference population to maximize accuracy of genomic breeding values (Calus, 2010; Pszczola *et al.*, 2012). However, matching of controls with cases on factors such as pedigree is essential to limit spurious associations in a case-control design. Knowledge of an optimal reference population design when observations are collected according to such a matched case-control design is lacking. Implementation of genomic selection in a horse population requires a considerable investment in the reference population.

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Summary

Summary

Insect bite hypersensitivity (IBH) is the most common allergic skin disease found in various horse breeds in countries worldwide and is caused by bites of tiny flying insects, *Culicoides* spp. Sensitive horses develop a severe itch after exposure to *Culicoides* spp. Affected horses rub and scratch themselves to alleviate the itch, which results in self-inflicted trauma. Welfare and economic value of affected horses is reduced and severely affected horses are sometimes euthanized. At present, there is no fully effective preventive or curative measure available. IBH is multifactorial in nature and therefore involves environmental as well as genetic factors. Breeding against IBH has the potential to permanently reduce prevalence. The aim of this thesis was to increase our understanding of the genetic background of IBH to be able to reduce prevalence in horse populations in the Netherlands.

Reducing prevalence of genetic disorders through breeding requires knowledge of genetic predisposition of breeding candidates, for which commonly breeding values are estimated. An increased knowledge of IBH associated environmental factors will contribute to greater breeding value precision. We identified and quantified risk factors for IBH in the Friesian horse and Shetland pony mare population (**chapter 2**). Mares were scored for IBH clinical symptoms by inspectors during annual obligatory foal inspections. Data were analyzed using a multivariable logistic regression model in each breed separately. IBH prevalence in the Friesian horse population was 18.2% and in the Shetland pony population 7.5%. The combined effect of month and year of scoring, region within the Netherlands and inspector were significantly associated with IBH prevalence in both populations. In Shetland pony mares, IBH prevalence significantly differed with withers height category and coat colour. Body condition score had a nearly significant overall effect; a high body condition score was related to an increased probability to show IBH.

Genetic variance should exist to be able to select against IBH and determines potential rate of genetic gain. Estimated variance components in Shetland pony and Friesian horse mares are described in chapter 3 and 4. In **chapter 3**, IBH observations collected during 2003, 2005 and 2006 on Shetland pony mares were analyzed. Roughly 25% of mares were scored more than once. IBH observations were corrected for year and month of score, climate, habitat, withers height category and age. Four generations of pedigree were considered and a permanent environmental effect was fitted. Estimated heritability on the observed binary scale was 0.08 (se = 0.02) and on the underlying quantitative scale 0.24 (se = 0.06). Repeatability was 0.30 (se = 0.02) and indicated that repeated observations on IBH clinical symptoms will improve accuracy of estimated breeding values. In **chapter 4**,

IBH observations collected during 2004 and 2008 on Friesian horse mares were analyzed. Roughly 10% of mares were scored twice and about 25% of the dams (n = 669) had more than 1 offspring in the data. IBH observations were analyzed using a Bayesian approach with a threshold animal model. The model included the age of the mare, year and month of IBH scoring, region and inspector as well as an additive genetic, permanent environmental and maternal effect. Heritability on the observed scale was 0.07 and on the underlying scale 0.16 (sd = 0.06). Repeatability was 0.89 (sd = 0.03) and the maternal effect was 0.17 (sd = 0.06). All parameters differed significantly from zero, although the animal model without maternal effect fitted the data better. IBH is a heritable phenotype in both the Friesian horse and Shetland pony population. Inclusion of repeated observations, a maternal effect and various other environmental effects like region will improve accuracy of IBH breeding values and thereby selection decisions.

Genome-wide association studies in Shetland pony mares and Icelandic horses are described in chapter 5 and 6. Genomic regions that contribute to genetic variance of IBH were identified and quantified. Controls were matched with cases on withers height category, coat colour, region and sex, and paternal half-sibs were sought to minimize population stratification and thereby spurious associations. IBH clinical symptoms were scored by an experienced veterinarian and a questionnaire was conducted to increase reliability of phenotypes. In **chapter 5**, single-nucleotide polymorphisms (SNPs) on 188 Shetland pony mares were analyzed using a single-SNP logistic model fitting an additive effect. Significant associations with IBH were detected on 12 chromosomes. Elimination of the unfavorable allele could reduce IBH prevalence to values between 4.4 and 6.8% in the Shetland pony population. We anticipated on a common genetic background, although breed differences in sensitivity to IBH are expected. Horses from many breeds in countries worldwide are affected and IBH prevalence varies between breeds, even within a similar environment (e.g. Friesian horse and Shetland pony mares, chapter 2). Validation of identified genomic associations in other horse breeds was therefore needed. In **chapter 6**, data on 200 Shetland pony mares and 146 Icelandic horses were analyzed. Breed-specific genome-wide association studies were performed. A threshold model was fitted and the Bayesian variable selection method Bayes-C identified associated genomic regions. A non-overlapping 1 Mb windows approach, accumulating effects of adjacent SNPs, was used to identify genomic regions explaining the largest percentage of IBH genetic variance. 13% of the genetic variance was explained by all SNPs in Shetland pony mares and 28% of genetic variance in Icelandic horses. Overlap in identified associated genomic regions between breeds would suggest interesting candidate regions for follow-up.

Analyses in both breeds identified genomic associations on chromosomes 3, 7, 11, 20 and 23. Associated genomic regions on chromosome 20 in both breeds were within 2 Mb from the equine lymphocyte antigen class II region. Identification of genes associated with IBH will contribute to our knowledge of its etiology and will increase efficiency of selection, prevention and therapeutic measures.

The general discussion, **chapter 7**, explored possibilities to reduce IBH prevalence in horse populations and illustrated implications of various phenotypes of IBH like clinical symptoms and results from *in vivo* and *in vitro* tests. IBH clinical symptoms are fully conditional on exposure to *Culicoides* spp. By setting specific requirements on IBH data collection, reliability of observed clinical symptoms will increase and thereby improve accuracy of breeding values, although diagnostic test results offer opportunities to improve selection decisions. Genetic gain of various selection strategies were simulated for IBH clinical symptoms and a diagnostic test (e.g. *C. obsoletus* specific IgE levels). Selection decisions were based on EBV, which included own performance, progeny performance or genomic data. Genomic selection outperformed selection on EBV that included own performance or progeny performance. However, implementation of genomic selection in a horse population requires a considerable investment in the reference population. Diagnostic test results like *C. obsoletus* IgE levels have the potential to increase genetic gain compared to selection for IBH clinical symptoms. Selection for IBH clinical symptoms should be based on testing at least 10 but preferably more progeny, accompanying strict selection in sires to achieve reasonable genetic gain (about 3% reduction in IBH prevalence per generation). Implications concerning future breeding against IBH were discussed as well.

Samenvatting

Samenvatting

Staart- en maneneczeem (SME) is een allergische aandoening die wereldwijd voorkomt bij paarden en pony's van verschillende rassen. De aandoening wordt veroorzaakt door beten van vliegjes van slechts enkele millimeters groot, genaamd knutten. Vrouwtjes knutten hebben bloed nodig voor het maken van eitjes en bijten daarom gewervelden, zoals paarden. In landen met een gematigd klimaat zoals Nederland, zijn knutten vooral actief rond zonsondergang in de periode april tot oktober. Dit is de reden dat SME in dergelijke landen een seizoensgebonden karakter heeft en in de winter niet optreedt. Beten van knutten kunnen een hevige jeuk veroorzaken waardoor paarden zich intensief gaan schuren wat kan leiden tot haarverlies, verdikte huid, schilfers, korsten en mogelijk open wonden en ontstekingen voornamelijk aan de staartwortel en manenkam (vandaar de naam). Het welzijn en de economische waarde van paarden met SME is verminderd. Paarden met zeer ernstige SME symptomen moeten soms worden afgemaakt. Momenteel is er geen effectieve behandeling om SME te genezen of te voorkomen. SME is een multifactoriële aandoening; naast de aanwezigheid van knutten zijn zowel factoren uit de omgeving van een paard als de genetische aanleg die bepalen of SME optreedt. Een uitvoerige beschrijving van SME wordt gegeven in **hoofdstuk 1**. Het doel van dit proefschrift was om het begrip over de genetische achtergrond van SME te vergroten om uiteindelijk het aantal paarden met SME in Nederland te verminderen.

Door middel van selectie van ouderdieren kan het aantal paarden met SME worden teruggebracht. Kennis van de genetische aanleg (uitgedrukt in de fokwaarde) voor SME van fokmerries en dekhengsten is noodzakelijk om gericht tegen SME te kunnen selecteren. Omdat het optreden van SME niet alleen wordt bepaald door de genetische aanleg maar ook door omgevingsfactoren zal een fokwaarde meer betrouwbaar zijn wanneer we bij de schatting ervan rekening houden met die omgevingsfactoren. De omgevingsfactoren zijn geïdentificeerd en gekwantificeerd in de Friese paarden en in de Shetland pony populaties (**hoofdstuk 2**). In beide populaties zijn symptomen van SME gescoord door inspecteurs gedurende de jaarlijkse, en verplichte, veuleninspecties. Het onderzoek wees uit dat 18,2% van de Friese merries en 7,5% van de Shetland merries SME symptomen vertoonden. Statistische analyse liet zien dat in beide rassen het percentage merries met SME significant verhoogd was in bepaalde regio's binnen Nederland zoals Gelderland en Noord-Brabant, en op bepaalde momenten (maand en jaar) van scoren. Effecten van regio en tijdstip hebben hoogstwaarschijnlijk te maken met de aanwezigheid van en daarmee blootstelling aan knutten. In Shetland pony's verschilde het

percentage merries met SME eveneens significant tussen hoogtemaatcategorieën en ook tussen vachtkleuren. Daarnaast was er ook een relatie tussen overgewicht en SME.

Er moeten genetische verschillen in gevoeligheid voor SME in een populatie bestaan om te kunnen selecteren tegen SME. Hoe groter deze verschillen, hoe strenger er geselecteerd kan worden en dus hoe sneller het aantal paarden met SME verminderd kan worden. Shetland merries werden gescoord voor SME gedurende 2003, 2005 en 2006. De verzamelde gegevens werden gebruikt voor het schatten van erfelijkheidsgraad en fokwaardes (**hoofdstuk 3**). Om een meer betrouwbare schatting van de fokwaarde van een merrie te krijgen, werd in de analyse van SME gegevens rekening gehouden met jaar en maand van scoren, hoogtemaatcategorie, leeftijd, en klimaat (regenval, aantal koude en warme dagen) en habitat (vegetatie en bodemtype) van de betreffende regio binnen Nederland. Vier generaties aan voorouders werden in de analyse meegenomen. De erfelijkheidsgraad van gevoeligheid voor SME was 24%. Dit betekent dat 24% van de verschillen in gevoeligheid voor SME tussen individuen kan worden verklaard door genetische aanleg. Geen enkele andere factor heeft een dergelijk groot effect. Een kwart van de merries was in meerdere jaren gescoord. Hierdoor was het mogelijk om de herhaalbaarheid van het vertonen van SME symptomen te bepalen. Een hoge herhaalbaarheid werd verwacht omdat bij gevoelige paarden ieder jaar SME symptomen optreden. De herhaalbaarheid was 30% en geeft aan dat herhaalde observaties aan hetzelfde paard de betrouwbaarheid van de fokwaarde verhogen. Gedurende 2004 en 2008 is SME gescoord bij Friese merries door inspecteurs. De gegevens zijn geanalyseerd met behulp van een statistisch model waarbij rekening gehouden werd met de leeftijd van de merrie, jaar en maand van scoren, regio en inspecteur (**hoofdstuk 4**). In Friese merries was de erfelijkheidsgraad van gevoeligheid voor SME 16%. Tien procent van de merries was in beide jaren gescoord, de herhaalbaarheid was 89%. Een kwart van de moeders had meer dan één nakomeling met een SME observatie. Nakomelingen van een moeder kunnen meer op elkaar lijken dan verwacht op basis van genetica bijvoorbeeld doordat ze als veulentje in dezelfde omgeving zijn opgegroeid. Een dergelijk maternaal effect op SME is onderzocht in de Friese paarden populatie en bedroeg 17%. Mogelijke oorzaken van een maternaal effect op de gevoeligheid voor SME zouden gerelateerd kunnen zijn aan bijvoorbeeld de melksamenstelling van de moeder, het management (voeding, stalling) van de eigenaar en omgeving gerelateerd via blootstelling aan knutten. Echter, dit maternale effect zal nader onderzocht moeten worden. Uit de analyses kunnen we concluderen dat erfelijkheid een rol speelt bij het optreden van SME in zowel de Friese paarden als

de Shetland pony populatie. De betrouwbaarheid van fokwaardes en daarmee selectiebeslissingen kan worden vergroot wanneer belangrijke omgevingsfactoren, maternaal effect en herhaalde observaties worden meegenomen in de schattingen. Erfelijkheid speelt dus een rol bij het optreden van SME. Maar het is niet bekend welke stukjes van het erfelijke materiaal (DNA) bijdragen aan de gevoeligheid voor SME. Als deze DNA stukjes geïdentificeerd zijn, dan kan er gerichter geselecteerd worden tegen SME en zal het aantal paarden met SME sneller verminderd kunnen worden. Het doel van het DNA onderzoek (hoofdstuk 5 en 6) was dan ook het identificeren van DNA stukjes die bijdragen aan de genetische verschillen in gevoeligheid voor SME. Voor het DNA onderzoek is bloed (met daarin DNA) verzameld van paarden met SME symptomen ("cases") en paarden zonder SME symptomen ondanks blootstelling aan knutten ("controls"). Cases en controls zijn zorgvuldig uitgekozen volgens een vooraf vastgesteld streng protocol om onjuiste resultaten te voorkomen. Het DNA van de cases werd vergeleken met het DNA van de controls op tienduizenden genetische merkers. Een genetische merker is een te herkennen stukje DNA en elke merker heeft zijn eigen specifieke plek op een chromosoom. De genetische merkers zijn gelijkmatig over de chromosomen verdeeld, zodat een groot deel van het erfelijke materiaal van een individu onderzocht kan worden. In het onderzoek kon een genetische merker drie vormen aannemen, bijvoorbeeld "AA", "AB" en "BB". Wanneer bijvoorbeeld alle cases "AA" hebben en alle controls "BB", dan kan er gezegd worden dat het DNA stukje rondom deze merker significant bijdraagt aan de verschillen in gevoeligheid voor SME tussen individuen. Om het aantal paarden met SME te verminderen, zouden in dit voorbeeld paarden met de gewenste "B" vorm als ouderdier geselecteerd worden, omdat alle individuen zonder symptomen "BB" hebben. Ongeveer 50.000 merkers zijn geanalyseerd in 97 Shetland merries met SME symptomen en 91 Shetland merries zonder SME symptomen (**hoofdstuk 5**). Meerdere DNA stukjes zijn geïdentificeerd die een rol spelen bij de gevoeligheid voor SME. Selectie voor de gewenste vorm van de genetische merker (in het bovenstaande voorbeeld "B" vorm genoemd) zou het percentage Shetland merries met SME kunnen verminderen van 7,6% tot waardes tussen de 4,4 en 6,8%. Er wordt verwacht dat dezelfde DNA stukjes verantwoordelijk zijn voor de gevoeligheid voor SME in meerdere paardenrassen, hoewel verschillen tussen rassen in gevoeligheid niet uit te sluiten zijn aangezien het percentage paarden met symptomen verschilt tussen rassen, zelfs binnen eenzelfde omgeving (bijvoorbeeld tussen Friese merries en Shetland merries in Nederland). Een bevestiging van de geïdentificeerde DNA stukjes in andere rassen was daarom noodzakelijk. Hiervoor werden wederom tienduizenden merkers geanalyseerd van 200 Shetland merries (103 met en 97

zonder SME symptomen) en 146 IJslandse paarden (73 met en 73 zonder SME symptomen) (**hoofdstuk 6**). Een overeenkomst in de geïdentificeerde stukjes DNA tussen de rassen geeft interessante kandidaat DNA regio's voor vervolgonderzoek. Overeenkomst werd gevonden in meerdere regio's, namelijk op chromosoom 3, 7, 11, 20 en 23. De geïdentificeerde DNA stukjes op chromosoom 20 liggen dichtbij het "equine lymphocyte antigen class II complex", waarvan al is vastgesteld dat het een rol speelt bij SME. Het vinden van genen die bijdragen aan de gevoeligheid voor SME zal de kennis over de oorzaken van SME vergroten. Daarmee kan de selectie ter vermindering van SME efficiënter gemaakt worden.
Het vertonen van SME symptomen is afhankelijk van blootstelling aan knutten, want zonder bloostelling zullen er geen symptomen van SME optreden ondanks een mogelijke erfelijke aanleg voor SME. Specifieke voorwaarden aan de gegevensverzameling, onder andere met betrekking tot deze blootstelling, kunnen de betrouwbaarheid van observaties aan SME symptomen en daarmee fokwaardes verhogen. De algemene discussie, **hoofdstuk 7**, beschrijft deze voorwaarden en ook mogelijkheden om selectiebeslissingen te verbeteren door het ontwikkelen van een diagnostische test (bijvoorbeeld het meten van bepaalde stoffen in het bloed). Voor verschillende selectiestrategieën werd berekend hoe snel het aantal paarden met SME zou verminderen. Selectiebeslissingen werden gebaseerd op fokwaardes. Bij het schatten van fokwaardes werd gebruik gemaakt van 1) 'eigen prestatie', waarbij SME gegevens of testresultaat van het paard zelf worden gebruikt, 2) 'prestaties' van nakomelingen, waarbij SME gegevens of testresultaat van nakomelingen van het paard worden gebruikt en 3) DNA informatie. Selectie met behulp van DNA informatie verminderde het aantal paarden met SME het snelst. Maar, het invoeren van selectie met behulp van DNA informatie in een fokprogramma vereist een aanzienlijke investering in een 'referentie populatie'. Dit is een grote groep individuen waarin de bijdrage van genetische merkers aan gevoeligheid voor SME wordt berekend. Na berekening van deze bijdrage van merkers in een referentie populatie kan voor een individu waarvan DNA aanwezig is, een fokwaarde worden geschat zonder dat daarbij een observatie aan SME symptomen noodzakelijk is. Selectie op diagnostische testresultaten zou kunnen zorgen voor een snellere vermindering van het aantal paarden met SME dan selectie op waargenomen SME symptomen. Selectie op waargenomen SME symptomen zou moeten worden gebaseerd op observaties aan minimaal 10, maar bij voorkeur 20 (of meer), nakomelingen. Wanneer er ook een strenge selectie in dekhengsten plaatsvindt, zal het percentage paarden met SME met circa 3% per generatie verminderen.

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Peer-reviewed publications

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About the author

Curriculum vitae

Anouk Schurink was born on the 30th of November 1982 in Doetinchem, the Netherlands. She was raised in several villages in the Achterhoek and obtained her high school diploma in 2001 at the Almende College, location Isala in Silvolde. During the same year she started her bachelor study at the Larenstein International Agricultural College in Deventer. She specialized in equine management. The subject of her bachelor thesis performed at the Studbook of the Royal Dutch Sport Horse was horse breeding in theory and practice. In 2005 she successfully obtained her bachelor degree. During the same year she started her master study in Animal Sciences and Aquaculture at Wageningen University with specialization Animal Breeding and Genetics. Her master thesis at the Animal Breeding and Genomics Centre focused on genetic parameters of osteochondrosis in Dutch Warmblood horses. In 2007 she graduated "cum laude", after which she was appointed scientific researcher at the Animal Breeding and Genomics Centre, Wageningen University. In 2008 she started her PhD research on a STW funded project entitled "Development of intervention strategies for insect bite hypersensitivity (IBH) in horses". Results of her PhD research are described in this thesis. Currently, she is working as a post-doctoral researcher at the Animal Breeding and Genomics Centre.

Curriculum vitae

Anouk Schurink is geboren op 30 november 1982 te Doetinchem en groeide op in meerdere dorpen in de Achterhoek, waaronder Gaanderen en Silvolde. In 2001 behaalde ze haar vwo diploma aan het Almende College, locatie Isala in Silvolde. Datzelfde jaar begon ze aan haar hbo studie Dier- en Veehouderij met als specialisatie Paardenhouderij aan de Internationale Agrarische Hogeschool Larenstein in Deventer. In 2005, na haar afstudeeropdracht bij het Koninklijk Warmbloed Paardenstamboek Nederland met als onderwerp fokkerij in theorie en praktijk, rondde ze haar hbo studie succesvol af. In hetzelfde jaar begon ze met haar master opleiding Dierwetenschappen aan Wageningen Universiteit. Voor het afronden van haar specialisatie Fokkerij en Genetica deed ze onderzoek naar de genetische achtergrond van osteochondrose in Nederlandse warmbloed paarden. In 2007 studeerde ze “cum laude” af, waarna ze als wetenschappelijk onderzoeker werd aangesteld bij de Animal Breeding and Genomics Centre, Wageningen Universiteit. In 2008 startte ze met haar promotieonderzoek op het door STW gefinancierde project “Ontwikkeling van interventie strategieën voor staart- en maneneczeem bij paarden”. De resultaten van haar promotieonderzoek zijn beschreven in dit proefschrift. Momenteel is ze werkzaam als postdoc bij de Animal Breeding and Genomics Centre.

Training and education

Training and education

The basic package (3 ECTS)

WIAS introduction course	2009
Ethics and philosophy of animal science	2010

Scientific exposure (15.8 ECTS)

International conferences

59th EAAP, Vilnius, Lithuania	2008
60th EAAP, Barcelona, Spain	2009
9th WCGALP, Leipzig, Germany	2010
62nd EAAP, Stavanger, Norway	2011

Seminars and workshops

Genetic resources in developing countries, Wageningen, the Netherlands	2008
Education in equine science in EU: new prospects, Vilnius, Lithuania	2008
Emerging vector borne viral diseases, Lelystad, the Netherlands	2008
Genetics and immunology of IBH in horses, Wageningen, the Netherlands	2009
Balans tussen fokkerij en biodiversiteit bij paarden, Lelystad, the Netherlands	2009
Genome-wide evaluation and GS, Wageningen, the Netherlands	2009
Genomics and animal breeding, Wageningen, the Netherlands	2011
15th QTL-MAS workshop, Rennes, France	2011
Healthy as a (sport)horse, Wageningen, the Netherlands	2011
Mini symposium on advanced genetics, Wageningen, the Netherlands	2012
Fokkerij en genetica connection days, Vught, the Netherlands	2008, 2010
WIAS science day, Wageningen, the Netherlands	2009 – 2012

In-depth studies (7.5 ECTS)

Epigenesis and epigenetics, Wageningen, the Netherlands	2008
Use of high-density SNP genotyping, Ames, Iowa, United States	2009
WIAS advanced statistics course, Wageningen, the Netherlands	2009
Statistical methods in animal breeding, Enaforsholm, Sweden	2009
Getting started with ASReml, Wageningen, the Netherlands	2009

Professional skills support courses (8.8 ECTS)	
Argumentation and scientific writing skills	2008
PhD competence assessment	2008
Supervising MSc thesis work	2010
Career assessment	2011
Lecturing	2012

Research skills training (8 ECTS)	
Preparing own PhD research proposal	2008
External training period, ISU, Ames, Iowa, United States	2011

Didactic skills training (9.7 ECTS)	
Lecturing	
Guest lectures genetic background of IBH, Utrecht, the Netherlands	2010, 2011
Review of RMC proposals, Wageningen, the Netherlands	2009
Supervising theses	
Supervising 3 MSc major theses	2009 – 2010
Supervising 2 BSc theses	2010

Management skills training (5.5 ECTS)	
Organization of scientific seminar on IBH, Wageningen, the Netherlands	2009
Membership WAPS council, Wageningen, the Netherlands	2009 – 2010

Training and education total	**58.3 ECTS**

Dankwoord

Dankwoord

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Shetland pony mare Twilight van Veldzicht (sire: Hose Ebony) and her foal Fabricius van Veldzicht (sire: Plesman van Veldzicht) on the cover were bred and are owned by Bert Dibbits and were photographed by Panya Sae-Lim. The photograph of *Culicoides* spp. on the cover was kindly provided by Nathalie van der Meide.

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