Tail Biting and Feather Pecking: Using Genomics and Ethology to Explore Motivational Backgrounds

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Abstract

It is well known that abnormal animal behaviour is affected by both environment and genetics. This thesis aimed to use behavioural observations as well as gene expression measurements to explore how animals that perform and receive tail biting (pigs) and feather pecking (laying hens) differ from individuals that are not involved in these behaviours.

In study I, the results suggested that tail biting is related to other abnormal behaviours. Pigs performing a high frequency of tail biting focused on abnormal behaviours that included oral manipulation (such as ear and bar biting), whereas those performing less tail biting showed a wider variety of different abnormal behaviours. In study II and III, many genes were differently expressed when neutral pigs were compared with tail biters and receivers, all housed in the same pen, as well as when compared to control pigs housed in a pen with no tail biting. This suggests that the neutral pigs had a phenotype that made them somewhat resistant towards performing and receiving tail biting. Behavioural data, in combination with information on the functions of these differently expressed genes, indicated that this difference in behaviour was due to the neutral pigs being less pig-directed in their behaviour. The focus on pigs which remain neutral in the outbreak is a new approach in tail biting studies. The gene expression data further suggested that selection for production may unintentionally have created pigs that perform and receive more pig-directed abnormal behaviour. In study IV, the functions of many of the 16 genes differently expressed between feather pecking hens, victims and control birds, support earlier hypotheses about feather pecking being linked to nutrition/feeding and immune mechanisms.

In summary, this thesis provides both behavioural characteristics and lists of genes that strengthen earlier reported results as well as give new suggestions about the biological mechanisms underlying tail biting and feather pecking behaviour.

Keywords: pig, poultry, tail biting, feather pecking, abnormal behaviour, animal welfare, genetics, gene expression

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Brunberg, E., Wallenbeck, A. & Keeling, L.J (2011). Tail biting in fattening pigs: Associations between frequency of tail biting and other abnormal behaviours. *Applied Animal Behaviour Science* 133, 18-25.

II Brunberg, E., Jensen, P., Isaksson, A. & Keeling, L.J. Brain gene expression differences are associated with abnormal tail biting behavior in pigs. Manuscript.

III Brunberg, E., Jensen, P., Isaksson, A. & Keeling, L.J. Behavioural and brain gene expression profiling in pigs during tail biting outbreaks - evidence of a tail biting resistant phenotype. Manuscript.

IV Brunberg, E., Jensen, P., Isaksson, A. & Keeling, L. (2011). Feather pecking behavior in laying hens: Hypothalamic gene expression in birds performing and receiving pecks. *Poultry Science* 90, 1145-1152.

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Abbreviations

5HT	5-hydroxytryptamine (serotonin)
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
EU	European union
GO	Gene ontology
HFP	High feather pecking
HP	High performer (tail biting)
HPA	Hypothalamus pituitary adrenal
HR	High receiver (tail biting)
IBD	Inflammatory bowel disease
KEGG	Kyoto Encyclopedia of Genes and Genomes
LFP	Low feather pecking
LIMMA	Linear models for microarray data
LP	Low performer (tail biting)
LR	Low receiver (tail biting)
mRNA	Messenger ribonucleic acid
NP	Non performer (tail biting)
NR	Non receiver (tail biting)
OCD	Obsessive compulsive disorder
P-HFP	High feather pecking line selected for production
P-LFP	Low feather pecking line selected for production
QTL	Quantitative trait loci
RMA	Robust multi-array average
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
TIM	Tail-in-mouth

1 Introduction

Abnormal animal behaviour, such as tail biting in pigs and feather pecking in laying hens, is wide-spread among farm animals. These behaviours are considered severe welfare problems and can be difficult to prevent even with proper housing and management. It is well known that behavioural traits are formed by the individuals' genes in interaction with the environment and previous experience. Therefore, knowledge about the genomics underlying abnormal behaviour may contribute to understanding why some individuals develop these behaviours, and so how they can be prevented.

In the studies described in this thesis, behavioural characterisation and brain gene expression in performers and receivers of tail biting (fattening pigs) and feather pecking (laying hens) were used to compare them to individuals not involved in these injurious behaviours.

The introduction starts with a short description of how animal welfare, genomics and behaviour are associated and continues with examples of different methods that can be used when studying behavioural genomics in farm animals. Further on, the abnormal behaviours tail biting in pigs and feather pecking in laying hens are described with emphasis on internal factors associated with the occurrence of these behaviours.

1.1 Welfare, genomics and behaviour

The main aim with genetic selection in farm animals has traditionally been to increase production and by that economic profit. This selection has been very successful and desirable production traits, such as growth, have increased significantly during the last decades. However, in addition to increased production levels, this intense selection also contributed to undesirable and harmful side effects, such as different production diseases (Rauw *et al.*, 1998).

The discussion about whether welfare related traits should be included in the selection is ongoing and most people probably agree that, for example, health traits should be addressed in the breeding goal.

Health is not the only trait with effects on animal welfare. Proper behaviour is central for animals to be able to adapt to their environment (Jensen, 2006). The behaviour of one individual also affects the welfare of the other group members. Aggression and injurious behaviours are examples of this. The behaviour of an individual is of course very much influenced by its surrounding environment. However, it is known that behaviour is also influenced by genes and it is suggested that the intense selection on production traits not only led to production diseases, but also changes in behaviour that can be detrimental for the welfare of the animals (Rydhmer, 2005).

Both the positive and negative sides of including behavioural traits in selection has been much debated (for example Turner, 2011; D'Eath *et al.*, 2010; Rodenburg *et al.*, 2010a; Mason & Latham, 2004; Kenttämies *et al.*, 2002; Jones & Hocking, 1999). But the reason for studying behavioural genetics is not only to be able to change behaviours through breeding. Understanding the genetic mechanisms underlying behaviour and genetic associations between production and traits with implications for animal welfare is of great importance (Jensen, 2010; Jensen, 2006). Behavioural genetics may also be looked upon as a tool to understand the biological mechanisms and hence the motivation underlying certain behaviours. This knowledge is interesting in itself, but may also be a key to decrease behaviours that are unfavourably linked to good animal welfare. The main aim with the present thesis was to use both genomics and ethology to explore biological mechanisms underlying two injurious behaviours in pigs and poultry; tail biting and feather pecking.

1.2 Methods to study behavioural genetics in production animals

Complex traits, such as behaviour, are influenced by environmental factors and are polygenic, i.e. influenced by many genes (Hofmann, 2003). Selection is one of the commonly used methods to determine to what extent behaviour is inherited (heritability) and how it is related to other traits. One of the classical behavioural selection experiments is the one described by Trut (1999), in which foxes were selected on the basis of their fear reactions to humans. Interestingly, when selected for low fear reactions, the foxes expressed almost dog-like behaviour as well as developing other physiological side traits often seen in the domestication process. Another example is the work by Kjaer *et al.* (2001), in which laying hens were selected for high and low feather pecking behaviour. This resulted in two lines that differed significantly in feather pecking behaviour in only two generations. The two lines are also frequently used in many feather pecking studies.

A genetic correlation between two different traits indicates they are influenced by the same genes. This can have important implications for breeding in production animals. As mentioned earlier, the intense selection on production traits has led to an unintentional selection on other traits with detrimental effects on animal welfare. This is not only true for production diseases, but also for behaviour (Rauw *et al.*, 1998). One example is injurious tail biting behaviour in pigs, linked to production traits such as back fat thickness (Breuer *et al*., 2005; Moinard *et al*., 2003).

Finding changes in the DNA associated to complex traits, such as behaviour, was a difficult task before the development of quantitative trait loci (QTL) analysis. This method is based on different types of markers spread throughout the genome and aims at finding regions (QTLs) in the genome that have polymorphisms associated to the trait of interest (see for example Flint & Mott, 2001 for a review). Examples of QTL studies concerning behavioural traits in production animals are temperament traits in cows (Guitérrez-Gil *et al.*, 2008), maternal infanticide in pigs (Chen *et al.*, 2009) and fear in poultry (Schütz *et al.*, 2004). One drawback with QTL studies is that it is often difficult to identify the gene(s) responsible for the association with the behaviour (Hofmann, 2003).

The expression of genes, and hence differences in the abundance of RNA molecules can be linked to differences in the DNA sequence. But gene expression is a dynamic process that is also very much influenced by environmental factors (Gibson, 2008). Hence, while QTL studies aim at finding differences in the DNA sequence that may be related to the function of that gene, gene expression studies look at the abundance of RNA molecules from specific genes. Differences in gene expression can be related to mutations, but that is not necessary, which contributes to that gene expression profiles can be different in different tissues. Studies regarding gene expression in production animals have increased in number and there are commercially available gene expression microarrays to measure genome wide gene expression in several of the production animals. Instead of the traditional molecular tools that allow only one or a few genes to be studied, gene expression microarrays can be used to monitor the levels of transcripts in the whole genome in a sample (Slonim & Yanai, 2009). This is useful for polygenic traits and traits for which it is difficult to apply a candidate gene approach (Konradi, 2005). The method is based on the immobilizing of probes (cDNA or oligonucleotides) on for example a glass slide. mRNA is isolated from the tissue of interest and, after colour labelling, it is allowed to bind to the probes on the array. The strength of the signal is then used to determine the mRNA levels from different genes (corresponding to the sequence of the individual probe; Konradi, 2005). The studies often result in very large data sets. These are statistically challenging and it is often a difficult task to determine which of the differently expressed genes that are most promising (Hofmann, 2003). On the other hand, the broader focus of microarray studies is the strength of global gene expression studies (Konradi, 2005).

1.3 Abnormal animal behaviour

When discussing so called abnormal behaviour, the first question to be answered is “what is normal and what is abnormal?”. The environment in which a population of animals live has a large effect on behaviour. Therefore, any abnormal behaviour that is influenced by the production environment may be shown by a majority of the animals. Hence, just because a large proportion of the animals in a certain population perform for example stereotypic behaviour, does not mean it can be regarded as normal. Instead, the norm (i.e. normal behaviour) must be behaviour performed in the natural habitat of the species (Keeling & Jensen, 2002). Even if this implies that the term abnormal may be slightly misleading since it can be a more or less normal reaction to an unnatural environment, the term “abnormal behaviour” will in this thesis be used according to the above definition.

Whenever an animal is kept under restrictive conditions, there is a risk for abnormal behaviours to develop and examples probably occur in all species kept for food production. The performance of abnormal behaviour is generally a sign that an individual has difficulties coping with its’ environment and hence is an indication that the performer is, or has, experience(d) reduced welfare. Abnormal behaviour directed towards other animals can cause injury, making them severe welfare problems also for the receiving animals.

That environmental factors have such a large impact on the development of abnormal behaviour has led to that many studies focus on how changes in the housing and management may decrease the problem. However, not all individuals in a group start performing abnormal behaviour, despite sharing the same environment and it seems likely that individual variation, such as genetic makeup, can predispose an animal to develop abnormal behaviour. This may also be one of the reasons why it is so difficult to prevent the behaviour with only environmental measures.

The difficulties in preventing these behaviours, together with their severe consequences for both welfare and economics, have in some cases led to procedures to physically change the animals. To prevent tail biting in pigs and feather pecking in laying hens, tail docking and beak trimming are often performed. According to EFSA (2007), over 90% of the pigs within the European Union are tail docked, even though routine tail docking is prohibited (EU Directive 91/630 EEC). Tail docking and beak trimming are criticized, not only because the procedures may cause neuromas and hence chronic pain (Simonsen *et al.*, 1991; Breward & Gentle, 1985), but also because it can be discussed if it is ethically justified to physically change animals to fit the production environment.

As a result of the above concerns, it is becoming increasingly important to understand the motivation and biological mechanism underlying these behaviours in order to prevent them. The key to this may lie in exploring the characteristics of performers and receivers of these behaviours and how they differ from other animals in the same environment (Edwards, 2006).

1.4 Tail biting in pigs

Tail biting is one of several abnormal behaviours seen in the domestic pig and is characterized by one pig orally manipulating and biting the tail of a pen mate. The term is used for behaviour ranging from gentle manipulation to severe cases of cannibalistic behaviour (Taylor *et al.*, 2010). Tail bitten pigs show inflammatory responses (Heinonen *et al.*, 2010), often have health problems and may have abscesses at slaughter with subsequent carcass condemnation (Munsterhjelm *et al.*, 2010; Kritas & Morrison, 2007; Martínez *et al.*, 2007; Walker & Bilkei, 2006; Valros *et al.*, 2004; Huey, 1996; Wallgren & Lindahl, 1996). It has also been shown that tail biting is more common on farms with health problems (Moinard *et al.*, 2003).

Research on tail biting has mainly focused on different environmental factors influencing the behaviour and the results indicate that it is of multifactorial origin (see review by EFSA, 2007). van Putten (1969) suggested that since pigs are highly explorative animals, the barren production environment does not fulfil the pig's behavioural need to root and chew, which is then redirected to the tails and ears of other pigs. This is also supported by a large number of studies emphasizing the positive effect of enrichment substrates, preferably straw, on tail biting behaviour (e.g. Zonderland *et al.*, 2008; Moinard *et al.*, 2003; Day *et al.*, 2002; Hunter *et al.*, 2001). Linked to explorative behaviour, is the suggestion that nutritional and metabolic factors may contribute to the development of tail biting. Factors such as restricted feeding/high feeding competition (Moinard *et al.*, 2003; Hunter *et al.*, 2001) and decreased levels of protein may also affect tail biting behaviour (Fraser *et al.*, 1991).

1.4.1 Some methodological considerations in tail biting studies

To estimate the prevalence of tail biting, most studies report observations on tail damage at abattoirs and very few direct observations of the behaviour itself (EFSA, 2007). This approach is useful to evaluate risk factors, but it overlooks the understanding of the performer (Edwards, 2006). Even though tail biting is very much influenced by environmental factors, it is not performed by all pigs on a farm, despite sharing housing conditions and management routines (Keeling *et al.*, 2004b). The performers vary in how much they participate in the behaviour during a tail biting outbreak with some being more pronounced tail biters (Zonderland *et al.*, 2011a; Van de Weerd *et al.*, 2005). This variation in tail biting behaviour among individuals housed in the same environment implies that other factors affect the development of the behaviour.

Since most pigs within the EU are tail docked, most published data concern pigs with docked tails (EFSA, 2007), despite the ban on tail biting as a routine procedure. This may be a problem since tail docking decreases tail biting behaviour to a large extent, making it more difficult to identify performers and non-performers. Furthermore, environmental factors have different effects depending on whether or not the tail is docked (Hunter *et al.*, 2001; Hunter *et al.*, 1999; McGlone *et al.*, 1992; Krider *et al.*, 1975) and how large part of the tail that is left also affects tail biting (Sutherland *et al.*, 2009).

Taylor *et al.* (2010) also raised the question of terminology. Since the term 'tail-biting' is used in different studies to describe everything from oral manipulation to actual cannibalistic behaviour, comparisons between studies are difficult. Severity scoring has mainly been based on different stages of tail damage (for example Heinonen *et al.*, 2009; Zonderland *et al.*, 2008; Valros *et al.*, 2004), even if some attempts have been made to classify the severity of the actual behaviour (see for example Schroder-Petersen *et al.*, 2003).

1.4.2 Sex

That castrated males seem to be more predisposed to being a victim of tail biting has been suggested based on observations of tail damage at abattoirs (Kritas & Morrison, 2007; Valros *et al.*, 2004; Hunter *et al.*, 1999; Wallgren & Lindahl, 1996; Penny *et al.*, 1981; Penny & Hill, 1974; Penny *et al.*, 1972) but also from on-farm studies (Walker & Bilkei, 2006; Kritas & Morrison, 2004). The reason for this may be that castrated males are less active than females (Hansen *et al.*, 1982) and hence an easier target (EFSA, 2007; Schroder-Petersen *et al.*, 2003).

Even if there is a general suggestion that females perform more tail biting behaviour, studies are inconsistent and the suggestion is not based on direct observations of females performing a larger amount of tail biting. Some studies that have used behavioural observations could not find any sex differences (Zonderland *et al.*, 2011b; Breuer *et al.*, 2003). The results that led to the hypothesis that tail biters are more often females are mainly based on records of tail damage and group composition. Schroder-Petersen *et al.* (2003) found that more tail in mouth (TIM) behaviour was performed in mixed sex groups than in single sex groups, and that females performed more TIM than they received and vice versa for males. Zonderland *et al.* (2010) could show that all-female groups of weaned piglets reached the point where 40% of the pigs in the pen had tail damage sooner than all-male groups and mixed sex groups. In the mixed sex groups, males had more tail damage than females. Although, at the end of the tail biting period, female and male single-sex groups both had more tail damage than mixed groups. Also Hunter *et al.* (2001) concluded that pigs from single sex groups had higher tail damage at slaughter compared to the ones housed in mixed sex groups. One study also showed that female piglets tended to have a higher motivation to manipulate a rope, possibly related to tail biting behaviour (Breuer *et al.*, 2003). Contradictory to the hypothesis suggesting that females perform more tail biting behaviour than males is data from Van de Weerd *et al.* (2005). Their results instead indicate that males were more likely to be fanatical tail biters (although no other sex differences could be found regarding tail biting behaviour). Taylor *et al.* (2010) suggested that males and females have different dietary needs and hence some pigs within the group will have dietary imbalances and may therefore be more likely to perform tail biting.

1.4.3 Breed differences and genetics

It has long been hypothesized that there is a significant effect of breed on tail biting behaviour (e.g. Penny & Hill, 1974). However, only a small number of studies addressed this question and the results are sometimes contradictory, perhaps due to differences in sample sizes, animal material, observation methods and environmental factors. No breed differences in tail biting behaviour were found by Lund & Simonsen (2000) comparing Danish Landrace and Duroc pigs. The pigs in this study showed very low levels of tail biting. No difference in tail biting behaviour was found by Guy *et al.* (2002) when comparing the progeny of indoor (Large White x Landrace) or outdoor (part Meishian or part Duroc) sows mated with Large Whites. Although, a Swedish study comparing over 3000 Swedish Landrace, Yorkshire and Hampshire boars, could show that Landrace boars were more often tail biters. Yorkshire pigs were found to be receivers more often, with Hampshire receiving less tail biting than both other breeds (Westin, 2000).

Breuer *et al.* (2005) and Breuer *et al.* (2003) addressed the question of whether or not tail biting is heritable in two studies. The first study tested the motivation to chew a rope, in which Durocs showed a higher propensity to chew and manipulate compared to Large Whites and Landrace pigs before weaning. No difference was found when observing actual tail biting behaviour in the home pen four weeks after weaning, but Duroc pigs performed more ear biting compared to Landrace pigs with the Large White intermediate. Duroc and Large White pigs also performed more of the pig-directed biting behaviour than Landrace pigs, but less belly nosing. In the second study, although only a tendency, a larger proportion of Landrace pigs were identified as tail biters compared to Large White pigs. Heritability was also estimated, with the performance of tail biting in Landrace pigs showing a heritability of 0.27, while it was not heritable in Large White pigs. Although it has been shown that genetics influences tail biting behaviour, no studies regarding molecular genetics associated with tail biting behaviour have been reported so far.

The results reported by Breuer *et al.* (2005) also indicated that there was a positive genetic correlation between tail biting and both lean tissue growth rate and back fat thickness. This is an unfavourable correlation since these are production traits that have been highly selected for in breeding programs. This was not the first study in which tail biting was found to be associated to back fat thickness. In an epidemiological case-control study, it was reported that when back fat thickness increased with 1 mm, the risk of tail biting decreased 1.5 fold (Moinard *et al.*, 2003). Moinard *et al.* (2003) suggested that this may be associated with the provision of straw, since pigs provided with straw have been found to have a higher back fat thickness (Beattie *et al.*, 2000). This is probably not the case since Breuer *et al.* (2005) later showed that these traits are associated also on a genetic level. Korte *et al.* (2009) also discussed that abnormal behaviour may be a consequence of modern selection. When focusing the selection on production traits, this may change the steroid balance, which can have impacts on sympathetic reactivity and corticosteroid concentrations (Korte *et al.*, 2009).

1.4.4 Tail biting and other abnormal behaviours

Tail biting is not the only abnormal behaviour expressed by pigs. Others frequently described in the literature include, for example, belly nosing, stereotypic bar biting and ear biting. A few studies have investigated the relationship between these different behaviours (see for example Bench & Gonyou, 2009; Beattie *et al.*, 2005; Hunter *et al.*, 1999). That there are some relationships is logical since abnormal behaviour is said to be a reaction to an environment that does not fulfil the animals needs. Results by Beattie *et al.* (2005) and Hunter *et al.* (1999) suggest that tail and ear biting are related. In the study by Beattie *et al.* (2005), tail biting was also positively correlated with nosing in the genital/belly region. Zonderland *et al.* (2011b) could not confirm this association, but could instead see that tail biters performed more manipulation behaviour directed towards enrichment devices, compared to control pigs. Bench & Gonyou (2009) distinguished between belly nosing and belly sucking and found that piglets performing high frequencies of belly sucking were less likely to be involved in tail biting behaviour in the grow-finishing phase, whereas the opposite was found for more general nosing behaviour. Rizvi *et al.* (1998) proposed a link between tail and vulva biting, since sow herds with vulva biting also had a higher occurrence of tail biting.

In summary, there is evidence of a link between other abnormal behaviour and tail biting, which implies either that pigs performing these behaviours have the same underlying predisposition or that the different behaviours are affected by the same environmental factors.

1.5 Feather pecking in poultry

Probably, the most observed and also investigated injurious abnormal behaviour in laying hens is feather pecking. It is characterized by a bird pecking and pulling on the feathers of another bird. It is said to exist in two different severity grades, with and without feather removal (gentle and severe

(Savory, 1995). The gentle form consists of more or less stereotypic pecking on the feathers, but without causing any injury and the victim usually does not react. The severe form includes pulling and sometimes pulling out and ingestion of feathers. This is painful for the recipient bird (Gentle & Hunter, 1991) and often results in a poor feather condition (Bilcik & Keeling, 1999). Moreover, exposed skin areas can predispose a victim of feather pecking to become a victim of cannibalism although there are types of cannibalism developing without feather pecking (Savory, 1995). Since it is the severe type of feather pecking that is regarded as a major problem in the egg production sector, the remainder of this thesis will mainly focus on this type.

Like tail biting, feather pecking is known to be influenced by several environmental factors (e.g. Drake *et al.*, 2010; Lambton *et al.*, 2010; Green *et al.*, 2000; Hughes & Duncan, 1972). Two main hypotheses regarding the underlying biological mechanisms of this behaviour exist, both suggesting a relationship to ground pecking behaviour (Savory, 1995). A well accepted hypothesis is that it has its background in foraging motivation (Blokhuis, 1986; Blokhuis & Arkes, 1984). The second hypothesis is that feather pecking is linked to dustbathing (Vestergaard *et al.*, 1993; Vestergaard, 1992).

1.5.1 Methodological considerations in feather pecking studies

In contrast to tail biting, feather pecking studies have during the later years focused on individual factors contributing to the behaviour. A large proportion of these studies have used lines known to differ in feather pecking behaviour. Two lines of White Leghorn that are widely used originate from two different breeding lines, included in a commercial selection program. Unintentionally, these two lines also differ in their level of feather pecking (Korte *et al.*, 1997). Two other lines, also commonly used in research, are the high and low feather pecking lines that were intentionally selected for and against feather pecking behaviour (Kjaer *et al.*, 2001). Both these lines are called high and low feather pecking lines (HFP and LFP respectively) in the literature. To distinguish between them, in this thesis these shortenings will be used for the lines directly selected on feather pecking. The lines selected for production will in this thesis be called production lines (P-HFP and P-LFP).

Studies using these lines have contributed significantly to the feather pecking research. However, when interpreting the results, consideration must be given to how/if the individual birds were phenotyped regarding feather pecking behaviour. In some of the studies, non-feather pecking characteristics of the lines were compared without performing any behavioural observations and hence not knowing the exact phenotype of the birds included (see for example Buitenhuis *et al.*, 2006; van Hierden *et al.*, 2002; Korte *et al.*, 1997). The two lines differ in many traits in addition to feather pecking behaviour, therefore the findings may not provide any direct evidence about the mechanisms underlying feather pecking. This may also be a drawback when using these lines in genetic studies. Genetic differences that are only indirectly related to feather pecking may be co-selected for in these lines. Hence, the results may be somewhat misleading, even if direct observations of the behaviour were performed.

1.5.2 Genetics

That genetics can predispose a bird to performing, and perhaps also receiving, feather pecks is rather well investigated. Different hybrids differ in how much they develop feather pecking (Uitdehaag *et al.*, 2008; Hocking *et al.*, 2004; Kjaer, 2000; Hughes & Duncan, 1972). Different lines were used in these studies and this, together with varying experimental environments, may have contributed to the not always consistent results. Although, all these studies showed an influence of hybrid on the behaviour and indicate that genetics is important for the development of feather pecking.

Since feather pecking has consequences for both bird welfare and economics, a possibility to select against the behaviour would be of benefit. Kjaer *et al.* (2001) described a successful selection experiment, in which a significant effect on the frequency of feather pecking could be seen after only two generations. This suggests that it is possible to include feather pecking as a trait in commercial breeding programmes.

To what extent feather pecking is influenced by genetics has been investigated by estimating heritability in a number of studies (e.g. Su *et al.*, 2005; Rodenburg *et al.*, 2003; Kjaer & Sorensen, 1997; Cuthbertson, 1980). Different animal material and different ways of estimating the frequency of feather pecking probably contributed to that the estimates vary between 0 (performed severe feather pecks in 6 and 30 weeks old chicks, Rodenburg *et al.*, 2003) and 0.56 for the proportion of feather peckers (Cuthbertson, 1980). This low to moderate heritability confirms that it is possible to select against the behaviour. Heritability for receiving pecks has been estimated and was found to vary between 0 (Rodenburg *et al.*, 2003) and 0.22 (Kjaer & Sorensen, 1997).

The molecular genetics of feather pecking behaviour is also rather well studied. The first QTL studies were reported by Buitenhuis *et al.*, (2003a and b). In these studies, a cross between the two earlier mentioned White Leghorn lines selected for production, but differing also in feather pecking behaviour were used. Behavioural phenotyping and a QTL analysis of the F2 generation resulted in three suggestive QTLs for performing gentle feather pecks and one significant for severe feather pecking (Buitenhuis *et al*., 2003b). It has also been suggested that being a victim of the behaviour could be affected by the genetic makeup. Four suggestive and one significant QTL have been associated with receiving gentle feather pecks (Buitenhuis *et al*., 2003a). In contrast to the Buitenhuis *et al*. (2003) studies, Jensen *et al.* (2005) used an F2 generation from an intercross between Red Junglefowl and White Leghorn. One suggestive QTL was found. The same birds were used by Keeling *et al.* (2004a) who found a very significant QTL associated with poor plumage condition. This QTL was found to have the causative gene *PMEL17*, encoding a protein known to control plumage colour and a dominant allele gives rise to the dominant white colour in chickens (Kerje *et al*., 2004). It was shown that pigmented birds were more vulnerable to receive pecks (Keeling *et al*., 2004a) and the gene also seems to influence other behavioural traits (Karlsson *et al.*, 2011; Nätt *et al.*, 2007). In conclusion, the performed QTL studies indicate that several genes affect feather pecking behaviour, each with a small effect.

The sequencing of the chicken genome provided further opportunities to perform studies using a single nucleotide polymorphisms (SNP) assay. Such a study was performed by Biscarini *et al.* (2010) who used 9 different genetic lines to perform an across-line SNP association study. It was suggested that the direct effect of the individual on its own feather condition reflect susceptibility to be a victim. The associative effect of the genotype of the cage mates on the individual‘s plumage condition would reflect the genes predisposing an individual to become a feather pecker. That there were more significant associations using the latter model, fits well with earlier results suggesting that genetics influences performers more than the victims. The function of many of the genes suggested an involvement of the serotonergic and immune system. A candidate gene approach was instead used by Flisikowski *et al.* (2009), indicating that differences in *DRD4* and *DEAF1* may be associated with feather pecking. These two genes encode proteins with important functions in the dopaminergic and serotonergic systems, respectively.

As mentioned earlier, not only polymorphisms on the DNA level may affect behaviour, but also differences in the transcriptome (gene expression). Brain gene expression differences in high and moderate feather peckers from the HFP line were investigated by Labouriau *et al.* (2009) and 456 genes were expressed differently between the two bird categories. Hughes & Buitenhuis (2010), however, could not find any differently expressed genes in the HFP line compared with the LFP one. Instead, they focused on the reduced variance in expression in the HFP line. Several genes with roles in nervous system development and immune mechanisms were correlated to the level of feather pecking and showed a negative skewness in the control population, indicating that they might have been targeted by selection favouring HFP birds.

In summary, genetics in interaction with the environment clearly contributes to birds performing and receiving feather pecking. Several genes probably have effects on the behaviour, each with a small effect. Moreover, some of the studies suggest involvement of neurotransmitters and the immune system, which fits well into the studies described in the next section.

1.5.3 Physiology and coping

A coping strategy predisposes how an individual responds to environmental challenges and consists of behavioural, physiological and neurobiological characteristics (reviewed by Koolhaas *et al.*, 1999). In many species, two extremes in coping strategies exist, the proactive and reactive strategy. Animals using the first strategy react towards a threat with an active fight/flight response while reactive individuals react with passive avoidance. Animals with the proactive strategy seem to be more vulnerable to develop behavioural pathologies such as stereotypic behaviour (Korte *et al.*, 2009; Koolhaas *et al.*, 1999). Therefore some studies have hypothesised that differences in coping strategy may underlie feather pecking behaviour (van Hierden *et al.*, 2002; Korte *et al.*, 1999; Korte *et al.*, 1997).

In several studies, hens from two lines selected for production, but also differing in feather pecking behaviour were used. It was found that birds from the P-HFP line had lower basal levels of corticosterone (van Hierden *et al.*, 2002; Korte *et al.*, 1997) and higher plasma nor-adrenalin levels in response to manual restraint (Korte *et al.*, 1997). They also had lower heart rate variability, (Korte *et al.*, 1999) than P-LFP birds, suggesting that the P-HFP birds were pro-active. The P-HFP hens also had lower serotonin and dopamine turnover compared to birds from the P-LFP line (van Hierden *et al.*, 2002). It has also been found that birds selected directly for and against feather pecking behaviour have different levels of plasma serotonin (Buitenhuis *et al.*, 2006). Although, it should be noted that in the many of these mentioned studies, no direct observations of feather pecking behaviour were performed, so the physiological measures could not be associated with the individual behavioural characteristics. Both the HFP/LFP and P-HFP/P-LFP lines differ in many traits in addition to feather pecking behaviour, therefore the findings do not provide any direct evidence about the mechanisms underlying feather pecking.

When associating the same physiological measures with the actual feather pecking behaviours, the results are sometimes contradicting. Neither Buitenhuis *et al.* (2004), Jensen *et al.* (2005) nor Kjaer & Guemene (2009) could find any altered corticosterone levels in performers of severe feather pecking. Although, the data indicated that performers of gentle feather pecking (Buitenhuis *et al.,* 2004) and victims (Jensen *et al.,* 2005) had lower corticosterone levels. On the other hand, van Hierden *et al.* (2004) found that birds from the P-HFP line injected with a serotonin (5-HT) receptor antagonist (and hence decreased 5-HT turnover) performed more feather pecking behaviour compared to non treated animals. It has also been found that an injection with the dopamine D2 receptor antagonist haloperidol decreased feather pecking behaviour in ISA brown chickens (Kjaer *et al.*, 2004). Hence, it still seems likely that the dopaminergic and serotonergic systems influence feather pecking behaviour.

1.5.4 Immune mechanisms

That the health of the victims of feather pecking can be affected is well known. However, recent studies also suggest that immune mechanisms may predispose an animal to perform feather pecking. It has been suggested that the same genes may influence both feather pecking and antibody response (Buitenhuis *et al.*, 2004) and that immunological parameters may change as a consequence of selection for a high frequency of feather pecking (Buitenhuis *et al.*, 2006). It has also been shown that groups of young bird challenged with antigen human serum albumin (HuSA, mimicking airborne immune challenges) later had significantly more feather damage (but less cannibalistic vent pecking) compared to unchallenged birds (Parmentier *et al.*, 2009).

1.5.5 Associations to body weight and production traits

The body weight of the individual hen is not clearly associated to feather pecking behaviour, since studies report contradictory results. In the two lines selected for a higher or lower frequency of feather pecking, the HFP line seems to be heavier (Kjaer *et al.*, 2001) while other studies found that feather pecking and low weight were genetically associated (Kjaer & Sorensen, 1997). The results from an intercross between Red Junglefowl and White leghorn hens showed that female feather peckers, in comparison to non-feather peckers, grew faster and started laying eggs earlier (Jensen *et al.*, 2005).

The most important selection traits in laying hens are probably those related to the actual egg production and quality. The LFP birds were found to lay more eggs and had a greater total egg mass, while the HFP line had a higher egg weight (Su *et al.*, 2006). A genetic correlation could not be shown (Buitenhuis *et al.*, 2004).

Jensen *et al.* (2005) could also distinguish certain physiological traits that were different in victims of feather pecking since they seemed to grow faster and were heavier. Interestingly, Buitenhuis *et al.* (2005) reported that the same genes may have implications for receiving gentle feather pecking at an early age as well as for body weight at 10 weeks of age. Hence, it seems likely that victims are not selected randomly and that physiological traits, such as body weight, may be predisposing. These results may also help explain the results from Kjaer *et al.* (2001) suggesting that birds from the HFP selection line are heavier, since birds from this line by definition also receive more pecks than the LFP one. This again emphasises the importance of characterising the birds on an individual level rather than only comparing the lines.

1.5.6 Feather pecking as a model for human psychological disorders?

Animals, mainly mice, are frequently used as models for human diseases, including behavioural disorders. Due to its' compulsive characteristics and possible association to the serotonergic system, van Hierden *et al.* (2004) suggested that feather pecking might be equivalent to obsessive compulsive disorder (OCD) in humans. OCD is characterized by thoughts (obsessions) and behaviours (compulsions) that are ritualized, for example excessive washing or hair plucking (trichotillomania) (Kuzma & Black, 2004). van Hierden *et al.* (2004) especially highlighted the similarities between feather pecking and trichotillomania.

Kjaer (2009) suggested that feather pecking is a hyperactivity disorder. Birds from the HFP line are much more active than the LFP birds. Furthermore, it was suggested that a disturbance in the dopaminergic system may be the link between increased locomotion and feather pecking.

1.6 Similarities between tail biting and feather pecking

Tail biting and feather pecking have some obvious common features. Both are so called injurious abnormal behaviours directed towards other animals in the group. They vary in severity, ranging from rather gentle manipulation, without the victim reacting, to actual cannibalistic behaviour and major responses by the victim as a feather is removed or the tail is damaged by a bite. The injuries they both cause, lead to decreased animal welfare and economical losses. As with other abnormal behaviour, they are said to be linked to a production environment that does not fulfil the animal's needs and is therefore regarded as an indication of decreased welfare also for the performers of the behaviour. Their biological backgrounds are believed to be a redirection of behaviours related to a motivation to forage and explore. Both tail biting and feather pecking are difficult to control and avoid, therefore it is common practice to physically change the animals to prevent them performing the behaviours i.e. tail dock and beak trim. Moreover, individuals sharing the same environment show a great variation in how much they perform/receive the behaviour. These behaviours must therefore be affected also by individual internal differences, and are likely to have a genetic background. Both also seem to be linked to production traits.

2 Aims of the thesis

The overall aim of this thesis was to use both gene expression and ethological approaches to further investigate the biological mechanisms and hence the motivation underlying feather pecking behaviour in laying hens and tail biting in pigs. This knowledge can hopefully ultimately be used to decrease these injurious behaviours. Moreover, by carrying out the studies on commercial farms, it was possible to use animals with the same genetic makeup and in housing conditions commonly used in animal production. The specific aims were:

- To use observational data to evaluate the link between tail biting and other abnormal behaviours in pigs to investigate the presence of a common motivational background. Furthermore, to develop a severity scoring system for tail bites, such as the one used for feather pecking, and to investigate whether different types of pigs differ in the severity of performed and received tail bites.

- To explore how brain gene expression in performers and receivers of tail biting and feather pecking differ from animals not involved in the behaviours. The intention, by studying somewhat similar abnormal behaviours in different species, was to compare the possible underlying mechanisms and motivations involved in these behaviours.

- To compare behaviour and brain gene expression in pigs not involved in either performing or receiving tail bites, despite being housed in a pen with an ongoing outbreak of tail biting, with pigs in a pen with no tail biting behaviour.

3 Materials and methods

3.1 Animals and housing

The studies were carried out on either a privately owned farm in Finland producing pigs for slaughter (study I, II and III) or a private farm with laying hens in Sweden (study IV). The animals were therefore housed and managed according to the different farm's regular procedures and according to Finnish or Swedish animal welfare regulations.

3.1.1 Tail biting in pigs (study I, II and III)

These studies were performed on a privately owned farm in the south of Finland raising approximately 800 slaughter pigs according to an 'all-in-all-out' production system. The pigs were of Finnish Yorkshire x Finnish Landrace x Duroc (x Hampshire) cross and arrived at the farm when they weighed approximately 25-30 kg (10 weeks of age). According to the Finnish legislation, male pigs cannot be castrated later than eight days after birth and tail docking is forbidden. Therefore all males were castrated long before arriving at the farm and all pigs had intact tails.

The farm consisted of six stables in two buildings. Pen and group sizes, ventilation system, pen equipment and feeding regimes varied both between and within the individual stables. All pens had a partly slatted floor. All pigs were housed in groups (mainly mixed gender) with group sizes varying between seven and 26 pigs and with a maximum stocking density of 0.65 m^2 per pig (for pigs weighing between 85 and 110 kg). There were water nipples in all pens, but feeding regime varied with group size. Pigs in the smaller groups (less than 11 pigs/pen, two of the stables) were fed a commercial feed for slaughter pigs twice a day, while the pigs in larger groups were fed the same feed *ad lib*. For enrichment, small amounts of peat were scattered on the floor daily.

3.1.2 Feather pecking in laying hens (study IV)

The study was performed on a poultry farm in the south of Sweden housing Lohmann selected leghorn laying hens. The birds arrived from the rearing farm at point of lay and all had intact beaks. One room, with 40 320 hens in 5 040 cages were used for the study. They were housed in furnished cages with 8-10 birds in each cage (minimum 0.06 m^2 per hen according to Swedish legislation) which were provided with perches, nest box and sand bath. Commercial layer feed and water were provided *ad lib*.

3.2 Behavioural observations

3.2.1 Associations between tail biting and other abnormal behaviours (study I)

Behavioural observations were carried out during four observation periods during May to October 2009. Observations were performed in five of the six stables during the first two observation periods. The pens were emptied, and then observations were carried out in four of the six stables during the two last observation periods. The youngest pigs included in the study were observed three days after arriving at the farm (approximately 10 weeks of age) and the oldest 11 weeks after arrival (approximately 21 weeks of age).

To be able to observe pigs that varied in their tail biting behaviour, both pens with expected tail biting problems and pens in which no tail biting was expected were chosen in each compartment. This selection was performed on the basis of tail status, short behavioural observations or after consulting the caretaker. The pigs in the selected pens (in total 742 pigs in 58 pens) were individually marked with colour on the back and observed for 2x30 minutes, either on the same day or on two consecutive days, once in the morning and once in the afternoon. The identity and sex of individuals performing and receiving mild, moderate and severe tail biting, tail in mouth, bar biting (only performer), ear biting, mounting and “other abnormal behaviours”, were recorded using all occurrence sampling.

3.2.2 Brain gene expression in tail biting pigs (study II and III)

The same observations as described for study I were used also in study II and III to select candidate tail biting pens (pens in which at least one pig was an active tail biter), candidate control pens (pens in which no or very little tail biting occurred) and to select candidate tail biter, receiver and neutral and control pigs for further observations. Both the neutral and control pigs were pigs not involved in tail biting behaviour as performers or receivers, but the neutral pigs were housed in a tail biting pen while control pigs were housed in a pen in which no or very little tail biting occurred. If candidate pens or individuals could not be identified with confidence after 60 minutes of observation (e.g. if the pigs were resting during the observation), those pens and the corresponding candidate individuals were observed for an additional 30 or 60 minutes to assure a correct categorisation.

In the next step of the observation procedure, the selected candidate tail biters, receivers and neutral pigs (selected from the tail biting pen) and control pigs (selected from control pens) were observed individually for 8x15 minutes distributed during one or two days. All occurrence sampling was used with the same ethogram as in the 30 minutes observation periods.

After this observation, matched trios or quartets of pigs were selected for tissue sampling. The aim was to select one tail biter, one receiver and one neutral pig from a tail biting pen and one control pig from a control pen in the same stable. In some of these matched groups either a neutral pig or control pig was missing. In total, seven complete quartets were selected, seven trios in which a neutral pig was missing and two trios in which a control was missing. Hence, 16 tail biters, 16 receivers, nine neutral and 14 control pigs were finally selected for tissue sampling. All pigs within a trio/quartet were matched for housing system, sex and age.

3.2.3 Feather pecking in laying hens (Study IV)

The behavioural observations were performed when the birds were between 41 and 49 weeks old. The aim from the beginning was to select all three bird categories (peckers, victims and neutrals) from the same cage, but it was not possible to find a neutral bird, i.e. an individual that did not perform or receive feather pecks in a feather pecking cage (i.e. a cage where an outbreak of feather pecking had occurred). A candidate feather pecking cage and adjacent control cage (i.e. a cage with no apparent feather pecking behaviour) were therefore selected based on the birds' plumage condition. All birds in the feather pecking cage were individually marked with leg rings and then observed in their home cage after 30 minutes of habituation to the observer. During the observations, the performer and receiver of all severe feather pecks (forceful pecks often involving feathers being pulled out and the victim reacting by e.g. vocalizing, turning around or running away) were recorded. These observations were performed to determine which individuals were candidate peckers and which were candidate victims. Birds in one cage were therefore observed as long as necessary to be able to determine this, but always between 30 and 150 minutes.

After this, the candidate pecker and victim, as well as two randomly selected birds from the adjacent control cage (candidate control birds), were marked with colour on the head. Each bird was then observed in their home cage for 30 minutes, where all performed/received severe feather pecks were counted. In this step performed/received bouts of gentle feather pecks were also noted. This observation was repeated three more times so that each bird was observed for two hours distributed across two days, both in the morning and in the afternoon. If the control birds performed or received feather pecks they were excluded and new candidate controls selected. In total, 209 birds were marked and observed.

After these observations, matched trios (and one quartet since one group included two peckers) of birds were selected for tissue sampling. In total 34 hens in 11 trios were selected.

3.3 Tissue sampling

3.3.1 Study II and III

One to three days after the last observation, tissues from the selected pigs in a trio/quartet were collected between 7.00 and 13.00 h. To minimize the stress for the pigs, they were sedated in their home pen using an intramuscular injection of midazolam (approximately 0.5 mg/kg), followed by butorphanol (0.2 mg/kg) and ketamine (10 mg/kg). When the pig was sleeping, it was carried to an adjacent building and euthanized with pentobarbitals (approximately 20 mg/kg). When no corneal reflex was shown, the head was removed and the brain dissected. The hippocampus, striatum, limbic cortex, prefrontal cortex, thalamus and hypothalamus were frozen in liquid nitrogen, transported on dry ice and preserved in $-80^{O}C$. The carcasses were transported to Helsinki University were they were autopsied and also the tail damage was scored using a five graded scale (0=no damage, 1=mild damage, superficial wounds, 2=moderate damage, deep wounds/moderate infection, 3=severe damage, part missing or severe infection and 4=abscesses).

3.3.2 Study IV

Two to five days after the observations, between 8.30 and 11.30 h, the selected birds were euthanized. They were taken to an adjacent room and quickly feather scored using a four point photographic scale where score 1 is naked and 4 fully feathered (Tauson *et al.*, 2005). After this, the bird was injected with pentobarbitals in the wing vein or/and in the abdomen until it was dead. The head was removed and the hypothalamus dissected. The brain areas were preserved as in study II and III.

3.4 RNA isolation and microarray hybridization (study II-IV)

Qiagens RNeasy lipid tissue mini kit (Applied Biosystems, Valencia, CA, USA) was used and the protocol followed when isolating RNA from both pigs and hens. The hypothalamus was used for the hens and the hypothalamus and prefrontal cortex for the pigs. Since the pig brain samples were much larger than needed for the RNA isolation, they were pulverized in liquid nitrogen prior to homogenization in Qiazol, and only a small fraction (as recommended by the manufacturer) of the pulverized brain area was used. The RNA concentration for each sample was estimated using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the quality with Agilent 2100 Bioanalyzer system (Agilent Technologies Inc, Palo Alto, CA, USA).

For the gene expression analysis, Affymetrix GeneChip® expression arrays were used and the GeneChip® 3' IVT Express Kit Manual (PN702646 Rev1, Affymetrix Inc, Santa Clara, CA, USA) followed. The biotinylated fragmented cRNA that is used in the hybridization was prepared using 250 ng (study II and III) or 2 µg (study IV) total RNA from each sample. The arrays were then hybridized for 16 hours in 45^{O}C and rotated at 60 rpm. Finally, the arrays were washed and stained using the Fluidics Station 450 and scanned with a GeneChip® Scanner 3000 7G, according to the GeneChip® Expression Wash, Stain and Scan Manual (PN 702731 Rev2).

For study II, samples from 29 pigs (11 tail biters, 10 receivers and 8 neutral pigs) were chosen for the microarray hybridization and in study III, samples from 12 pigs. For the laying hens, from the original 34 birds, 11 peckers, 10 victims, 10 controls were finally used for the hybridization, since the RNA samples from one trio were destroyed.

3.5 Data analysis

3.5.1 Behavioural data

Study I and III

In study I, each pig was assigned to a performer category and to a receiver category. These categories were based on the number of performed and received tail bites per 60 minutes of observation. In the three performer categories, non-performers (NP, n=643) performed zero tail bites, the low performers (LP, n=79) performed between one and four tail bites and high performers (HP, n=20) performed more than four tail bites. The three receiver categories were based on received tail bites: non receivers (NR, n=559) did not receive any tail bites, low receivers (LR, n=133) received one or two tail bites and high receivers (HR, n=50) received three or more tail bites. The frequency of performed and received abnormal behaviours during the 60 minutes of observations was compared between the different performer and receiver categories respectively.

In study III, all neutral and control pigs housed in the 12 matching tail biting and control pens were included in the behavioural analysis. In this study, a pig was regarded as a neutral pig or control if it did not perform any tail bites and received at most one. This resulted in 60 neutral pigs from the six tail biting pens and 99 control pigs from the six control pens. The frequencies of performed and received abnormal behaviours were compared between these two categories of pigs.

As earlier described, some pens were observed for more than 60 minutes if a candidate performer and receiver (for the gene expression study) could not be reliably identified within the observation time. When all pigs were included in the analysis in study I, only the two first performed observations were used. However, since the selection of pigs for the subsequent tissue sampling in study III was based on the two last performed observation periods, these periods were used also for the analysis of the behavioural data in this study. However, only the pigs in one tail biting pen and matching control pen needed to be observed for more than 60 minutes in study III.

SAS software (version 9.2, SAS institute, Inc. Cory, NC, USA) was used to analyze the behavioural data in both studies using the procedures RANK, GLM (study I) and MIXED (study III). To transform frequencies for each behaviour into a normal distribution, Blom normal rank scores were used (e.g. for each behaviour, each pig was assigned a rank according to how much of that particular behaviour it had performed and, likewise, a rank for how much it had received).

The statistical model, when comparing tail biting behaviour between the sexes in study I, included sex, stable and group-pen nested within stable as fixed effects. In the model for comparing performer/receiver categories, the fixed effects included category, sex, stable and group-pen nested within stable. The effects of group size/composition, season and feeding routines were included in the group-pen factor. To adjust the pair-wise comparisons for multiple comparisons, the Tukey-Kramer method was used. In study III, the statistical model for comparing performed and received behaviours between the neutral and control categories of pigs included the fixed effects of category, sex, stable and group-pen nested within stable as well as the random effect of pair.

Study II and IV

In study II and IV, the rates of performed and received tail bites/feather pecks, as well as tail damage/feather scores were compared to confirm that the categories differed. A Kruskal-Wallis test was used in study II and a Mann-Witney U test in study IV. Minitab 15 Statistical solutions software was used.

3.5.2 Microarray data (study II, III and IV)

Normalization of the raw data from the microarrays was performed in either the software Expression Console (Affymetrix Inc, Santa Clara, CA, USA) (study II and III) or statistical computing language R (http://www.r-project.org) (study IV) with the robust multi-array average (RMA) method (Irizarry *et al.*, 2003; Li & Wong, 2001). All subsequent data analysis was performed using R with packages from the Bioconductor project (www.bioconductor.org). An empirical Bayes moderated t-test within the 'Linear models for microarray data' (LIMMA) package was used to search for differently expressed genes. P-values were adjusted with the method of Benjamini & Hochberg (1995) to correct for multiple testing

3.5.3 Enrichment analysis (study II and III)

To search for enriched gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways among the differently expressed genes in the three comparisons in study II and the comparison in study III, the DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov) was used. To be able to reduce the uncertainty of such an analysis, Huang *et al.* (2008) recommend that a gene list consisting of at least 100 genes is used. Very few transcripts were differently expressed in the prefrontal cortex and therefore only the gene lists from the hypothalamus were used. Since only two transcripts were expressed significantly different when comparing tail biters and receivers and only 32 transcripts differed significantly ($p<0.05$) in the tail biter vs. neutrals comparison in study II, genes with higher p-values (<0.2) were used to be able to include at least 100 genes. This resulted in 211 genes when comparing tail biters and receivers, and 314 genes when comparing tail biters and neutrals. In the receiver vs. neutral comparison in study II, 130 genes were differently expressed ($p<0.05$), and therefore only these genes were used. In study III, the gene list consisted of 107 differently expressed genes.

4 Results

The most important results are summarized in the following paragraphs, more details can be found in each paper.

4.1 Study I

Out of the 742 observed pigs, 87% were never observed to perform any tail bites. The remaining 99 pigs that performed tail bites performed between one and 60 tail bites during the 60 minutes of observation. Regarding received tail bites, 183 pigs (25%) received between one and 30 bites during 60 minutes.

In comparison with the LP pigs, the HP ones performed a higher proportion of severe tail bites and a lower proportion mild tail bites. All three performer categories (HP, LP and NP) performed different frequencies of tail in mouth, with the HP category performing most and NP least. The LP pigs performed more belly nosing and 'other abnormal' behaviour than NP, while HP only tended to carry out more 'other abnormal' behaviour than NP pigs. The HP pigs were more active ear and bar biters than the NP category, while the LP pigs performed more bar biting than NP. The results are illustrated in Fig 1.

Regarding received behaviours, a lower proportion of mild tail bites was received by HR pigs compared to LR pigs and there was a tendency for the opposite for received severe tail bites. All three receiver categories differed in the frequency of received tail in mouth behaviour, with NR pigs receiving least and HR most. The HR pigs received more 'other abnormal' behaviours than both other categories, and there was a tendency for the LR category to receive more than NR pigs.

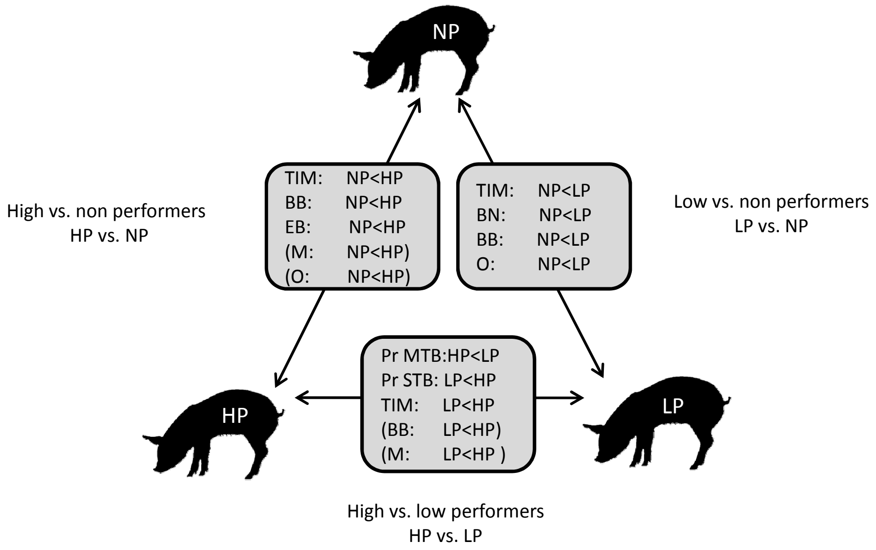

Figure 1. Abnormal behaviours in study I differing significantly (p<0.05) between the three performer categories; tail in mouth (TIM), bar biting (BB), belly nosing (BN), other abnormal O, ear biting (EB), mounting (M), proportion of mild tail bites (pr MTB), proportion of severe tail bites (prSTB). Behaviours within brackets indicate a tendency towards significance (p<0.1).

Differences in tail biting behaviour between males and females were also investigated, and there was a tendency for females to perform more severe tail bites than males.

4.2 Study II and III

The tail biters included in the microarrays (n=8) in study II performed on average almost 59 tail bites during the three hours of observation (0.33 bites/minute) while the receivers (n=7) received on average just over 22 tail bites (0.12 bites/minute). The neutral and control categories by definition did not perform any tail bites and did not receive more than one bite in the whole observation period. The 60 neutral pigs included in the behavioural analysis in study III performed significantly lower frequencies of tail in mouth, belly nosing and “other abnormal” behaviour compared to control pigs, whereas they performed more bar biting. This was partly also reflected in the frequencies of received behaviours since the pigs in the neutral category received less “other abnormal” and tended to receive less belly nosing, but significantly more mounting compared to the control pigs.

In study II, 32 genes showed a different expression in the hypothalamus when comparing tail biters and neutrals, 130 in the receiver vs. neutral comparison and two between receivers and neutral pigs. Not as many genes were differently expressed in the prefrontal cortex; seven genes in tail biters compared to neutrals and seven when comparing receivers and neutrals. When comparing the gene expression in the two control categories in study III (neutrals vs. controls), the expression level of 107 transcripts in the hypothalamus and 10 in the prefrontal cortex differed. Comparing tail biters vs. controls, nine transcripts were differently expressed in the hypothalamus and one in the prefrontal cortex. When comparing receivers with controls, three transcripts in the hypothamalmus and four in prefrontal cortex were differently expressed. When combining the data sets from the two studies, not all genes were differently expressed in only one of the comparisons. Many were similar between the two studies, and the majority of the genes that were differently regulated in several comparisons seemed to differ in neutral pigs compared to several other categories. A few genes were also differently expressed in both the hypothalamus and the prefrontal cortex.

4.3 Study IV

The feather peckers that were included in the microarrays pecked on average 1.16 pecks/minute and the victims received 0.26 pecks/minute. By definition, the control hens did not perform or receive feather pecks and they also had a better feather score than the other categories.

In total 11 transcripts were differently expressed when comparing peckers, victims and controls. Three of those were differently expressed in peckers compared to both controls and victims, one in controls compared to peckers and victims, four were unique for the pecker vs. control comparison and three unique for the pecker vs. victim comparison. Five additional transcripts tended towards significance in the pecker vs. victim comparison ($P<0.1$).

When comparing the results from the feather pecking study with the two tail biting studies, one gene (*COMP*) was differently expressed both in study III and IV (in peckers compared to victims of feather pecking and when comparing the neutral and control pigs).

5 General discussion

All studies in the present thesis were performed with the view that improved knowledge about how individuals involved in tail biting and feather pecking differ from other individuals may lead to an increased understanding of the underlying biological mechanisms. That different pigs perform different abnormal behaviours and that a grading of the severity of tail bites may be useful for both future research and for the farmer, is suggested by the results from study I. In study II and III, brain gene expression differences in tail biter, receiver, neutral and control pigs support earlier findings and provide new information that can help direct future studies. The results from study III also imply that there are pigs that seem to be resistant to developing pig-directed abnormal behaviour of any type. The gene expression results in study IV strengthen earlier hypotheses about feather pecking behaviour, such as an association to immune mechanisms and feeding motivation.

5.1 Behavioural categorization of tail biters and their receivers

5.1.1 Association between tail biting and other abnormal behaviours

That tail biters also performed a higher frequency of ear biting may be expected, since several earlier studies reported a link between the two behaviours (Beattie *et al.*, 2005; Hunter *et al.*, 1999; Blackshaw, 1981). This may imply that the same motivational system underlies these two behaviours. A second behaviour that was more common in tail biters compared to controls, and hence may also indicate a similar underlying motivation, was bar biting. Of the behaviours recorded in study I, these three associated behaviours (biting tails, ears and bars) were the three behaviours in the ethogram that by definition included biting. Hence, these pigs appear to be motivated to perform the action of biting itself.

Pigs performing a low frequency of tail biting were found to perform a higher frequency of belly nosing and "other abnormal" behaviour compared to control pigs, suggesting that there are different categories of tail biters. The high frequency biters seemed to direct their abnormal behaviour towards biting either pen mates or pen fittings, whereas pigs performing a lower frequency of tail biting included other types of pig-directed abnormal behaviours in their behavioural repertoire. In contrast to the present result suggesting that low frequency biters perform more belly nosing, Beattie *et al.* (2005) reported a direct positive correlation between the frequency of tail biting and belly nosing, although it was not very high (0.21). In their study, very few pigs seemed to perform any belly nosing and it might be that this apparent positive correlation was due to the contribution of the pigs not performing any tail biting at all (corresponding to the neutral pigs in our study).

In hens, it is suggested that there are traits predisposing some birds to become victims of social injurious behaviours (e.g. Keeling *et al.*, 2004a). If this is the case in pigs, it might be expected that the receivers of tail biting behaviour also receive more other abnormal behaviours, since the traits predisposing a pig to receiving different abnormal behaviour may be the same. This hypothesis is supported by the results of study I, in which it was found that pigs receiving high frequencies of tail biting also tended to receive more "other abnormal" behaviours. Why these pigs are more vulnerable to becoming victims of these behaviours cannot be answered by the results in this study. It has however been suggested that the lower activity level of these pigs may be a contributing factor (EFSA, 2007; Schroder-Petersen *et al.,* 2003).

5.1.2 Sex differences in tail biting behaviour

As mentioned earlier, it has been found that males have more tail damage than females and from this it has been suggested that males receive more tail bites (e.g. Walker & Bilkei, 2006; Van de Weerd *et al.*, 2005; Kritas & Morrison, 2004; Valros *et al.*, 2004; Hunter *et al.*, 1999; Penny *et al.*, 1972) . Zonderland *et al.* (2011b) studied tail biting outbreaks in 14 pens and identified the main biter or victim in each pen. Even if 11 out of the 14 victims were males, the difference was not significant. Neither could the results in this thesis support that male pigs receive a higher frequency of tail bites.

The only difference that could be found between males and females in this study was that females tended to perform a higher frequency of severe tail bites than males. Since the severity grading was based on the reaction, females seem to bite harder and hence they might cause more damage. This may partly explain why studies regarding the differences between males and females performing tail biting are somewhat inconsistent. Some studies did not find any difference between the males and females (Zonderland *et al.*, 2011b; Van de Weerd *et al.*, 2005; Breuer *et al.*, 2003; Blackshaw, 1981) and the ones that did find a difference draw this conclusion based mainly on comparisons of mixed or single-sex groups (Zonderland *et al.*, 2010; Schroder-Petersen *et al.*, 2003).

5.1.3 Using a severity scoring and tail biting frequency

One of the problems with many tail biting studies is the definition of the behaviour, since everything from gentle manipulation of the tail to real cannibalistic behaviour has been included in the definition (Taylor *et al.*, 2010). Schroder-Petersen *et al.* (2003) used the term tail-in-mouth behaviour (TIM) to describe the gentle manipulation of the tail, which does not involve actual biting and without the receiver reacting. However, this definition may be somewhat misleading since the tail biter can sometimes chew and even bite the tail of another pig without the receiver reacting. In this thesis, an attempt was made to classify the severity of tail bites, based upon the reaction of the receiver.

Except from characterizing the actual tail bites attempts have been made to classify different biters/outbreaks. Taylor *et al.* (2010) suggested that different outbreaks of tail biting may have different underlying motivations and suggested a division into three sub-categories: two-stage tail biting, sudden forceful tail biting and obsessive tail biting. However, it is a difficult task to try to categorize an outbreak when it has already started if the main difference between them is the underlying motivation and how the actual outbreak starts. Furthermore, even if the idea of different motivational backgrounds is useful for future scientific studies, it is of little use for the farmer. Another way would be to categorize different tail biters based on the frequency of performed and/or received tail bites observed during an outbreak. It is this type of classification which was performed in this thesis. A somewhat similar characterization was made by Van de Weerd *et al.* (2005), who classified some of the biters as "fanatical". But this was made on the basis of how persistent they were in their tail biting and activity level, and not on the frequency with which they performed tail biting.

When combining severity scoring and categorization based on frequency, it was found that the high performer pigs not only performed more tail bites per unit of time compared to the low performer category of pigs, but also that a higher proportion of these were severe tail bites. Hence, either the high performers are more vigorous in their biting or their receivers are more sensitive to having their tails bitten. These two possible reasons are of course related, if the high performers bite harder, the receiver will get an even more injured tail and hence be more sensitive. Irrespective of the reason, this knowledge may be useful for the farmer. One way to stop an already ongoing tail biting outbreak is to remove the biter. Although it has been reported that less than half of the farmers actually do this (Hunter *et al.*, 2001). The pigs performing high frequencies of tail biting are the ones that should be removed from the pen and looking at the victims' reaction may be helpful when identifying them.

5.2 Behaviour and brain gene expression in tail biting pigs

There are as yet no published studies on molecular genetics and tail biting. The aim with this study was to explore gene expression differences in tail biters and receivers compared to pen mates not involved in tail biting behaviour (neutral pigs). In addition to these pigs, which were housed together with at least one tail biter, control pigs that were not housed together with a tail biting pig were included. Gene expression in the hypothalamus and prefrontal cortex were compared in these four categories of pigs. Many of the genes that were differently expressed differed in more than one of the six comparisons. All genes were included in an enrichment analysis and some were subjects for a detailed literature and database search.

5.2.1 Are control pigs resistant to tail biting?

Together, the results of both gene expression and behavioural data in study II and III suggest that some pigs have a phenotype that is less pig-directed in their behaviour and, by that, resistant to both performing and receiving tail bites. These are represented by the neutral pigs in this study. Of all comparisons, the three comparing the neutral pigs to any of the other categories gave a relatively high number of differently expressed genes (between 32 in tail biters compared to neutrals and 130 in receivers compared to neutrals in the hypothalamus). In the three comparisons not including the neutral category, only a few genes were differently expressed (between zero in tail biters compared to receivers and nine in tail biters compared to controls). Hence, most differences were found when comparing the neutral pigs to the other categories of pigs. Moreover, of the genes that were differently expressed in more than one comparison, many differed in the neutral pigs compared to all other categories of pigs. This further implies that neutral pigs are fundamentally different from the others. One common dilemma with gene expression studies is whether differences in expression are a cause or a consequence to the phenotype (in this case involvement in tail biting). In the present studies, this was solved by including a pig category housed in a different pen, the control pigs. Since 18 genes were differently expressed in neutral pigs compared to all other categories, including the control pigs, these differences in expression cannot be an effect of the different pen environments. Neither can they be a consequence of the neutral pigs not performing/receiving tail biting.

The control pigs both performed and received more other abnormal behaviour compared to the neutral category, which further strengthens the hypothesis that the neutral pigs are fundamentally different. Since the pigs in this study shared the same farm environment, it would be expected that they develop abnormal behaviours. Despite not developing tail biting behaviour, the control pigs developed other abnormal behaviours. The only behaviour that the neutral category performed more of compared to the control pigs, was bar chewing, the only commonly occurring non-pig-directed behaviour. Hence, the neutral pigs showed some abnormal behaviour, but not to the same extent pig-directed.

The new hypothesis proposed in this thesis is that there is a group of pigs neither performing nor receiving tail biting (nor other pig-directed abnormal behaviour) despite being housed in the same environment as an ongoing outbreak of tail biting. It is proposed that this knowledge may help direct future research to prevent tail biting. Instead of focusing on why some pigs *do* perform or receive tail biting behaviour, it may be more beneficial to characterize the pigs that seem to have a behavioural and genetic profile which protects them from being involved in these behaviours.

5.2.2 Genes and gene ontology terms associated with neutral pigs

Among those 18 genes for which the neutral pigs in the tail biting pen had a different expression, there are some that may help explain why these pigs are resistant to the tail biting outbreak. As earlier mentioned, the neutral pigs were less pig-directed in their behaviour. This fits very well with the different expression of *GTF2I.* This gene, which was up-regulated in the hypothalamus in pigs of the neutral category compared to the other three categories of pigs, is suggested to influence social behaviour both in humans (Dai *et al.*, 2009) and mice (Sakurai *et al.*, 2011). It is one of many genes that are deleted in a disease called William-Beuren syndrome, giving not only physical but also behavioural anomalies (such as anxiety and hypersociability) (Pober, 2010). The main effect of the deletion of *GTF2I* seems to be hypersociability (Dai *et al.*, 2009). Sakurai *et al.*, (2011) reported that mice with a heterozygous knockout of this gene, which supports its effect on social behaviour. The heterozygous mice, with a lower expression of the gene, seemed to be more social towards unfamiliar mice. Interestingly, one test showed that they were less interested in a novel object compared to wild type mice. The pig-directed aspect of tail biting (compared to non-pig-directed abnormal behaviour such as bar chewing) makes the social versus object component angle of this relevant. Differences in social interactions could be linked to variation in tail biting. Moreover, if the neutral pigs are less pig-directed, this would be in line with them not performing and receiving abnormal behaviours directed towards or from another pig.

One of the main hypotheses about the motivation underlying tail biting behaviour is that it is related to the pig's motivation to explore. A gene that seems to have implications for a related trait, novelty seeking in humans, is *EGF* (epidermal growth factor) (Keltikangas-Järvinen *et al.*, 2006). This gene was found to be less expressed in neutral pigs compared to all other categories. Polymorphisms in this gene have been found to be associated with different temperament traits in Cloninger's temperament test, namely novelty seeking, reward dependence and persistence (Keltikangas-Järvinen *et al.*, 2006). One possible explanation for the involvement of this gene in these kinds of behaviours could be its effect on dopaminergic neuron development and neurotransmission (e.g. Iwakura *et al.*, 2011; Inazu *et al.*, 1999; Missale *et al.*1991). The possible involvement of this gene in tail biting is relevant, both due to its effect on behavioural traits and to its effect on the dopamine system. The latter is particularly interesting since dopamine is believed to influence feather pecking in laying hens (Flisikowski *et al.*, 2009; Kjaer *et al.*, 2004; van Hierden *et al.*, 2002).

Another gene that could be highlighted is *PDK4* (pyruvate dehydrogenase enzyme 4), that was up regulated in neutrals compared to all other categories of pigs. This mitochondrial protein is important for metabolism of glucose and energy (reviewed by Sugden & Holness, 2003), but is also linked to different production traits in pigs (Lan *et al.*, 2009). In a study by Lan *et al.* (2009) the gene was found to be more expressed in skeletal muscles from the Meishan breed compared to Yorkshire. The same study could also show that a polymorphism affected lean to fat ratio, intramuscular fat, muscle water content and tended to affect back fat thickness. This is noteworthy since tail biting is known to be both genotypically and phenotypically linked to reduced back fat thickness (Breuer *et al.*, 2005; Moinard *et al.*, 2003). Korte *et al.* (2009) argues that the selection for production traits may make the animals more vulnerable to developing abnormal behaviours due to underlying changes in steroid balance, sympathetic reactivity and corticosteroid concentrations. Knowledge about genetic and phenotypic correlations between production traits and injurious behaviours is very important to be able to include production traits in the breeding goal of pigs without risking the welfare of the animals.

That such a large proportion of the genes were differently expressed in the neutral pigs compared to all other categories of pigs was reflected also by the enrichment analysis. Several terms regarding neuron development were significant in all three comparisons included in the analysis (neutrals vs. tail biters; neutrals vs. receivers and neutrals vs. controls). A link between abnormal behaviour and the expression of genes involved in nervous system development was suggested by Hughes & Buitenhuis (2010) who investigated gene expression variation in feather peckers. This again highlights similarities in the possible underlying mechanisms for tail biting in pigs and feather pecking in poultry.

5.2.3 Differently expressed genes and pathways in tail biters and receivers

Among the significant gene ontology terms from the enrichment analysis, there were also those significant in only one comparison. Two worth mentioning when comparing tail biters to neutrals, are the two KEGG pathways ‘viral myocarditis’ and ‘host allograft rejection’. By definition, many of the genes in these pathways are probably involved in different immune mechanisms. It might have been expected that genes involved in immune mechanisms would be differently expressed between receivers and neutrals/controls, since injuries and infections following tail biting would be expected to affect gene expression. However, in this study the two KEGG terms were significant when comparing tail biters with neutral pigs. That abnormal behaviours can be linked to immune system has been suggested previously in feather pecking hens (Biscarini *et al.*, 2010; Hughes & Buitenhuis, 2010; Parmentier *et al.*, 2009; Buitenhuis *et al.*, 2006; Buitenhuis *et al.*, 2004). Tail biting is more common on farms with health problems (Moinard *et al.*, 2003) and illness can of course make an animal more vulnerable to receive these kinds of behaviours. Moreover, health problems may also trigger tail biting due to a changed neurotransmitter balance and changes in nutritional needs (Taylor *et al.*, 2010).

Since wounds and infections following tail bites can be expected to influence gene expression, it is not surprising that the genes included in the term “wound healing” were enriched in receivers compared to neutral pigs. However, effects of tail damage probably contribute to making other genes and ontology terms significant in receivers compared to neutrals difficult to interpret.

5.2.4 Gene expression differences in the neutral and control pigs

Since environment and stress clearly affect gene expression in pigs (Poletto *et al.*, 2006), the difference in environment between neutral and control pigs were expected to affect gene expression. The genes that were differently expressed only between neutral and control pens (and not between the neutral category and other pigs in the same pen) may likely be affected by the potential stressful situation of being housed in a tail biting pen. Many of the gene ontology terms significant in only this comparison were different homeostatic processes. One definition of stress is that it is a situation that is threatening or may threaten the homeostasis of an individual (see a recent review by Chrousos, 2009). These situations activate the hypothalamic-pituitary-adrenal (HPA) axis to release corticosteroids which have an effect on different processes such as metabolism, immune functions and development (see Herman *et al.*, 2003 for a review). In an earlier performed study, plasma calcium tended to be higher in pigs in control pens compared with pigs in tail biting pens (Holmgren *et al.*, 2004). Therefore, of the significant GO terms, the term ‘cellular calcium homeostasis’ may be of certain interest.

However, it is important to remember that gene expression is highly influenced by the environment. And since the environment in the specific pen may contribute to whether a tail biting outbreak occurs or not, small environmental differences in the specific pen can influence the expression of genes without being directly associated to the tail biting outbreak. It is known that the activity level in pens with tail biting is higher (Zonderland *et al.*, 2011b; Statham *et al.*, 2009; Keeling *et al.*, 2004b) and more feed is left in the feed trough (Wallenbeck *et al.*, 2010).

GHRL, encoding ghrelin-obestatin preprotein, was one of the genes with functions in homeostatic processes that was up-regulated in the neutral pigs compared to controls. This is in accordance with the fact that both emotional and physical stress seems to increase plasma ghrelin in rodents (Kodomari *et al.*, 2009; Kristensson *et al.*, 2006; Nishizawa *et al.*, 2006). Although, it’s most well-known function is as an appetite regulating hormone which is increased during fasting (see review by Hillman *et al.*, 2011), which could indicate that the neutral pigs eat less compared to control pigs. Moreover, the gene *COMP* that was among the differently expressed genes in feather pecking hens compared to receivers was also more expressed in the neutrals compared to controls. That the same gene was significantly differently expressed in both the feather pecking and tail biting study is especially interesting, even if it was differently expressed in different comparisons in the two species.

5.3 Gene expression in feather pecking laying hens

Relatively few transcripts were differently expressed in one or more of the three possible comparisons between feather pecker, victim and control birds in study IV (11 with $p<0.05$ and 5 with $p<0.1$). Therefore it was not possible to perform any enrichment analysis. Instead, the literature for all genes was reviewed for possible associations between the gene functions and feather pecking behaviour. The results from this literature search are presented in the next paragraphs.

Contradictory to expectation, only one transcript was found to be significant when comparing victims with control birds. This gene (*GTF2H5*, a general transcription factor), was also differently expressed in the peckers compared to the controls. This implies that the difference was due to the environmental factor of being housed in a cage with an outbreak of feather pecking. It also allowed the conclusion that plumage condition did not significantly influence gene expression in this study, which was also unexpected. The fact that victims and controls showed little difference in gene expression also implied that feather peckers could be compared with victim birds. This was useful since the aim from the beginning was to select complete trios from the feather pecking cage, but this was not possible. When comparing peckers and victims, the environmental effect of the different cage environments is removed.

5.3.1 Immune mechanisms

It has previously been proposed that feather pecking and immune mechanisms may be linked (Biscarini *et al.*, 2010; Hughes & Buitenhuis, 2010; Parmentier *et al.*, 2009; Buitenhuis *et al.*, 2006; Buitenhuis *et al.*, 2004). This suggestion is supported by the results from the present study since many of the differentially expressed genes are implicated in immunological processes. Two of the genes known to influence immune responses in different ways are *LAG3* (lymphocyte activation gene 3) and *TNFSF15* (tumour necrosis factor ligand superfamily, member 15) (Takimoto *et al.*, 2005; Triebel, 2003). These two genes differed in peckers compared to victims and controls, respectively. Furthermore, the gene with the highest fold change in peckers compared to both controls and victims, *ABCB1* (ATP-binding cassette, subfamily B, member 1, encoding multi drug resistant protein 1), is believed to have possible roles in immune mechanisms. The main function of this protein is as a transporter crossing the blood brain barrier where it can influence extrusion of proinflammatory cytokines (Kooij *et al.*, 2009). Another gene approaching significance in peckers compared to victims was *MAPK8* (mitogen-activated protein kinase 8), involved in production of cytokines as well as T-cell differentiation (Constant *et al.*, 2000; Dong *et al.*, 1998). Hence, the present study strengthens the proposed association between feather pecking and the immune system. As stated previously, it is also of interest since the enrichment analysis in the tail biting study indicated that genes with immune functions may be implicated also in this behaviour.

5.3.2 Intestinal inflammation, osteoporosis and feeding motivation

Two immune modulating genes, *ABCB1* and *TNFSF15*, are both said to affect intestinal inflammation and intestinal bowel disease (IBD) (Thiebaut *et al.*, 2009; Brant *et al.*, 2003; Panwala *et al.*, 1998). It is tempting to speculate whether possible discomfort in the gut could influence eating motivation and perhaps also feather pecking, since feather pecking is proposed to be redirected ground pecking behaviour (Blokhuis, 1986) and is linked to feather eating behaviour (McKeegan & Savory, 1999; Savory, 1995). An imbalance of nutrients in the provided feed can increase feather pecking behaviour (e.g. Ambrosen & Petersen, 1997; Hughes & Duncan, 1972). It is therefore noteworthy that some forms of IBD can result in nutrient deficiencies, partly following a decreased absorption in the intestines. Therefore, the disease is often associated with osteoporosis (Pigot *et al.*, 1992). This link is worth mentioning since Jensen *et al.* (2005) found an association between weak bones and feather pecking and suggested a resource allocation to production traits in feather peckers. All these links and functions of the genes would therefore fit well into the general consensus that feather pecking is strongly linked to foraging motivation.

Another possible link to feeding motivation is the function of *MAPK8.* A deficiency of its protein product in the central nervous system seems to affect feed intake as well as body mass and energy expenditure in mice (Sabio *et al.*, 2010). The protein is suggested to have implications in insulin resistance (Cho *et al.*, 2001) which is also the case for *AKT2* (v-akt murine thyroma viral oncogene homolog 2) (Velloso *et al.*, 2008). Both were close to significant in the comparison between peckers and victims.

There may also be associations between behavioural pathologies (such as feather pecking) and immunology, intestinal inflammation and glucose homeostasis. As mentioned earlier, van Hierden *et al.* (2004) suggested feather pecking to be a model for OCD in humans. OCD may in some cases be mediated by immune mechanisms (reviewed by da Rocha *et al.*, 2008). Moreover, depression seems to be more common among IBD patients (Walker *et al.*, 2008) as well as being linked to a disturbed glucose metabolism (reviewed by Hundal, 2007).

5.3.3 Comparison with other gene expression studies

This study was not the first investigating gene expression in feather peckers. That very few transcripts were differently expressed, is in line with the results from Hughes & Buitenhuis (2010), who did not find any transcripts that were differently expressed when comparing a high (HFP) and low (LFP) feather pecking selection line. In the study by Labouriau *et al.* (2009) whole brain gene expression in high and moderate feather peckers in the HFP line was compared. It is remarkable that the same gene, *LAG3* involved in immune responses, tended to be differently expressed in the present study and was differently expressed in the one by Labouriau. The differences between the studies are many (animal material, brain area and feather pecking frequency for example) and the role of this gene in feather pecking behaviour therefore deserves further investigation.

5.4 Comparing tail biting and feather pecking and suggestions for future studies

Understanding the motivational background and individual differences underlying abnormal behaviour is important in order to be able to prevent it. The data obtained from the studies presented in this thesis both provided new insights about these two abnormal behaviours, as well as strengthening some earlier proposed hypotheses. Mechanisms, pathways and genes that may be important for the development of abnormal behaviour, and in some cases also common for the studies II, III and IV, are discussed below and illustrated in Figure 2.

In the studies presented in this thesis, the gene *COMP* encoding cartilage oligomeric matrix protein, was among the differently expressed genes in both the tail biting and feather pecking studies. The protein is an extracellular matrix protein known to cause the dwarfing condition pseudoachondroplasia (Hecht *et al.*, 1995). It was however differently expressed in different comparisons: in feather peckers compared to victims and in neutrals compared to control pigs. Unfortunately, the possible implications of this gene on the development of abnormal behaviour are not known. Although, it seems justified to further explore the possible function the expression of this gene has in abnormal behaviour, or vice versa.

One of the most important findings in this thesis is that the neutral pigs seemed to differ in both behaviour and gene expression in comparison to tail biters, receivers and control pigs. Hence, these pigs seem to have a phenotype that is somewhat resistant to tail biting, a resistance that seems to be related to being less pig-directed in their behaviour. We therefore suggest that in future studies it may be worthwhile focusing on why some pigs *are not* involved in tail biting behaviour, as opposed to the earlier focus on why pigs *are* involved. The results also emphasize how important it is to select neutral animals from the actual tail biting pen when wanting to study individual differences underlying the behaviour. Studies aiming to confirm this, as well as studies investigating if there are such resistant animals in different species with other abnormal behaviours, would be useful. To test this approach in feather pecking would be especially interesting since the two behaviours have many characteristics in common. But it would demand observations of the actual pecking behaviour as well as other abnormal behaviours. To be able to select neutral hens that are housed together with a feather pecker, a larger group size than the ones used in study IV must probably be used.

In general, tail biting research should continue to focus much more on individual differences predisposing an animal to perform and/or receive abnormal behaviours as has been done in feather pecking research. As a complement to gene expression studies, and as done in feather pecking research, a polymorphism study with the aim of finding mutations in genes associated with the behaviour would provide more knowledge about how genetics can influence tail biting. In study II, it was suggested that differences in social and exploratory behaviour could contribute to the fact that the neutral pigs were not involved as tail biters or receivers. That a motivation to explore is important for the development of tail biting has been suggested before (EFSA, 2007; van Putten 1969) and this may be investigated with for example a novel object or open field tests. Such tests have earlier been performed in feather pecking studies (e.g. Rodenburg *et al*., 2010b; Uitdehaag *et al*, 2008; Albentosa *et al*., 2003). Differences in social motivation between controls and performers/receivers could also be tested. As social motivation has been suggested to be linked to feather pecking (Rodenburg *et al*., 2004), this would be another shared motivation with implications on both behaviours.

The association between immune defence and feather pecking has been suggested previously (Biscarini *et al.*, 2010; Hughes & Buitenhuis, 2010; Parmentier *et al.*, 2009; Buitenhuis *et al.*, 2006; Buitenhuis *et al.*, 2004) and the data presented in this thesis support this. In pigs, there are also links between diseases and tail biting (Moinard *et al.*, 2003). In enrichment analysis, the two significant KEGG pathways “allograft rejection” and “viral myocarditis” may indicate that genes with possible immune effects have implications on the difference between tail biters and neutral pigs. Hence, immune mechanisms may be associated to both these behaviours.

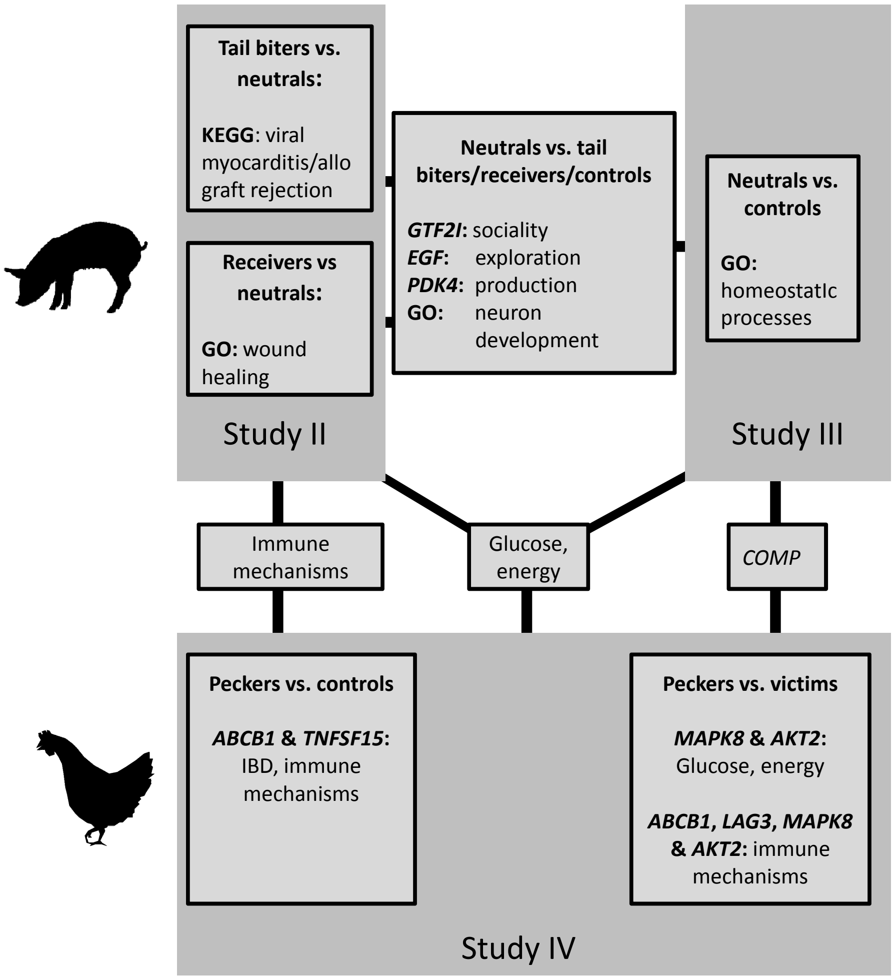

Figure 2. Possible common biological mechanisms and pathways between the two pig studies (study I and III) and the chicken study (study IV). Gene names are indicated by an italic font, KEGG refers to KEGG pathways and GO to gene ontology terms.

5.4.1 A relationship between production, coping style, stress response, immune mechanisms and abnormal behaviours

The suggested link between production traits and abnormal behaviour has been suggested to be due to that selection for higher production influences the coping style of the animal (Korte *et al.*, 2009). That intense selection on production traits may lead to an increase in abnormal behaviour has been suggested in general (Korte *et al.*, 2009) and the association between back fat thickness and tail biting (Moinard *et al.*, 2003; Beattie *et al.*, 2005) and between body size, egg production traits and feather pecking (Su *et al.*, 2006; Kjaer & Sorensen, 1997) support this. That the gene *PDK4* (known to influence fat content in pigs; Lan *et al.*, 2009) was differently expressed in neutral pigs compared to tail biters and receivers, as well as to pigs from the control category also strengthens this. It further suggests that this selection may have influenced both performing and receiving tail bites as well as other pig-directed abnormal behaviours. To investigate this association between abnormal behaviour and selection on production traits more thoroughly in feather peckers is a proposed area for further research.

The personality (i.e. coping style) of an individual influences stress response and also the vulnerability to develop abnormal behaviour (reviewed by Korte, 2009). Stress and immune response are known to be closely related and are discussed in the field of psychoneuroimmunology (see reviews by Pruett, 2003; Marsland *et al.*, 2002; Yang & Glaser, 2002). Exactly how stress may mediate immune functions and disease is still under investigation, but it is generally thought that chronic stress suppresses immune functions, but acute stress may instead enhance immune response (reviewed by Dhabar, 2009). If a connection between coping, stress, immune system and abnormal behaviour exists, it could be speculated that the intense selection on production traits may influence both behaviour and immune functions through stress response/coping style (Figure 3). This possible link may be explored in future studies by comparing coping strategies, production traits and immune mechanisms in performers and receivers of tail biting and feather pecking.

In a study by Yoder *et al.* (2011), Landrace pigs were more active during a performance test than Duroc, Chester White and Yorkshire and also had lower back fat thickness. A low, but significant, negative correlation was also found between temperament score and back fat thickness, indicating that the more reactive pigs were leaner.

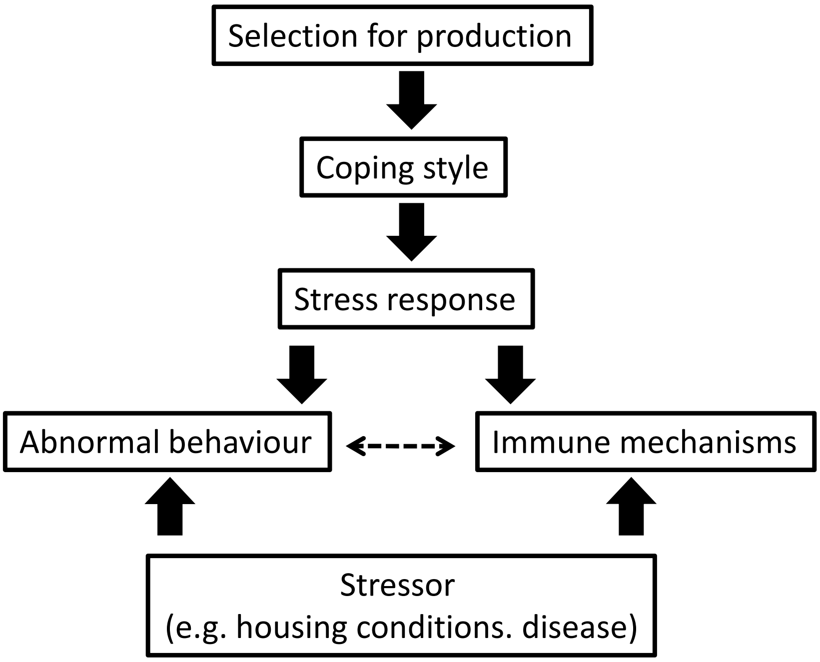

Figure 3. Possible associations between selection pressure on production, coping style and individual stress response. Coping style and stress response may influence both abnormal behaviour and immune mechanisms, which also seems to be linked.

Nutritional factors contribute to both tail biting (e.g. Moinard *et al.*, 2003; Hunter *et al.*, 2001; Fraser *et al.*, 1991) and feather pecking (reviewed by van Krimpen *et al.*, 2005). It is therefore interesting that genes with functions in glucose and energy metabolism were differently expressed in both tail biters (*PDK4*) and feather peckers (for example *MAPK8*). In summary, tail biting and feather pecking has many characteristics in common and it is probably beneficial to use somewhat similar research strategies when investigating the two behaviours.

5.5 Methodological considerations

5.5.1 Sex, breed and age

Hormones affect feather pecking (Hughes, 1973) and there are sex differences in tail biting (e.g. Kritas & Morrison, 2007; Valros *et al.*, 2004; Hunter *et al.*, 1999; Wallgren & Lindahl, 1996; Penny *et al.*, 1981; Penny & Hill, 1974; Penny *et al.*, 1972). The choice to use only female birds in this study was rather obvious as these are the ones used in egg production and they also perform a higher frequency of feather pecks (Jensen *et al*., 2005).

Regarding the tail biting pigs in study I, both females and males were used and we did not find any clear difference in tail biting behaviour between males and females. In study II and III, tissues from both females and males were collected, but all groups (trios or quartets) were balanced for gender. The main reason for not performing separate analyses for the males and females, as originally intended, was that we ended up with rather few samples suitable for the data analysis. Therefore, separate analyses for males and females would not have been biologically relevant due to the low number of replicates. On the other hand, it could be argued that both males and females are used in the production and often mixed in the same pen. Therefore, the most important biological mechanisms and pathways would be those relevant for both females and males and those should be captured by combining the data sets for the different genders.

Breed seems to have an effect on both tail biting (Breuer *et al*., 2005; Westin, 2000; Penny & Hill, 1974) and feather pecking (e.g. Uitdehaag *et al.*, 2008; Hocking *et al*., 2004; Kjaer, 2000; Hughes & Duncan, 1972). Although, it seems unlikely that the underlying biological motivation is different in different breeds, even if it is possible that the specific genes differently expressed may vary between breeds. In this study, we wanted to use commercial farms and they were chosen on the basis of the frequency of abnormal behaviours, not breed. This is also the reason for using a cross in study I-III. This cross is among the most commonly used in Finland. In study IV, we chose not to use the two existing lines differing in feather pecking behaviour (either selected for production or directly on feather pecking). First, these lines are widely used and many of the earlier genetic studies performed on feather pecking behaviour already used them. Secondly, studies of these lines may be somewhat misleading. The HFP lines are by definition also more vulnerable to be victims and it might have been difficult to distinguish which differences are related to the selection on the performance and which on the increased vulnerability to be a receiver.

Regarding the age of the animals, this is known to influence tail biting (EFSA, 2007). In the studies presented in the present thesis, we aimed at finding a large enough variation in behaviour to be able to identify performers/receivers as well as controls. Differences in age will most likely affect gene expression. In study II and III, this may lead to an increased variation within category (tail biters, receiver and neutral pigs) since many of the trios/quartets were of different ages. Although, within trios pigs were matched for age and this was taken into account in the analysis. As with the effect of breed, it is questionable if the basic underlying biology predisposing an animal to performing and receiving certain behaviour differ with age. In the feather pecking study all selected birds were of approximately the same age.

5.5.2 Euthanasia and tissue sampling procedure

When including gene expression in a study, the method of euthanasia is very likely to affect the results. In the pig studies, our only option was lethal injection since the brain needed to be intact for the dissection. We also wanted to avoid transporting the pigs to a slaughter plant and aimed at minimizing the time between euthanisation and freezing of the tissues to avoid RNA degradation. The reason for choosing the injection option also for the laying hens was to treat pigs and hens as equal as possible.

Regarding the dissection techniques, the earlier feather pecking gene expression studies used whole brains. In the pig studies, the hypothalamus and prefrontal cortex were used. The disadvantage with using only a specific area of the brain is, of course, that it is not possible to see expression differences in other areas of the brain. On the other hand, the risk with using the whole brain is that expression differences in small specific areas are diluted.

5.5.3 Microarrays: verification and interpretation of the results

It is often argued that gene expression differences identified with microarrays should be verified with methods like qRT-PCR (Konradi, 2005). However we chose not to perform a technical validation in the present studies. The Affymetrix platform is robust and technical variation is much smaller than biological variation. Moreover, biological validation of results should preferably be performed on independent samples, which were unavailable in this case. The fact that the results are not validated on independent data should be taken into consideration when performing studies based entirely on the data from the present studies.

A general concern when using gene expression to explore the biological background to certain traits is that it is very difficult to know if the different expression of a gene is a cause or an effect of the trait of interest, in this case tail biting or feather pecking. By including both neutral and control pigs in the tail biting studies it was easier to distinguish between cause and effect. As discussed by Hofmann (2003), another drawback with these types of studies is that it is difficult to determine which of the differently regulated genes are the most promising. To solve this in study II and III, the focus was on the genes that were differently expressed in more than one of the comparisons. An enrichment analysis was also performed to get an indication about which genes might be the most promising. In the feather pecking study (study IV) this was not a large problem since so few transcripts were differently expressed it was possible to go through them all thoroughly.

5.5.4 Choosing control animals

In study III, it was shown that the neutral pigs not only differed in gene expression and behaviour from tail biters and receivers, but also from the control pigs, housed in pens without tail biting outbreaks. Moreover, the differences between performers and receivers compared to the control pigs selected from control pens were few. Therefore, one of the conclusions from this study is the importance of choosing neutral/control pigs from the actual tail biting pen. This is an important consideration for future studies.

In study IV, the control birds had to be selected from an adjacent cage. The intention was to perform the selection of birds as in study II, i.e. a feather pecker, victim and a neutral bird from the same cage. However, it turned out to be impossible since all birds, except for the feather pecker, in a feather pecker cage were severely pecked. Another option would have been to perform this study in a loose housing system, but the large number of birds in these systems would have made the behavioural observations more complicated. This may although be considered in future studies.

It could be argued that the control birds in study IV were not optimal controls since they were housed in another cage and we know that neutral and control pigs differed. However, there are some differences between the results between pigs and poultry that to some extent justify the different selection of control animals. First, in study IV there was only one transcript that differed in expression between control birds and victim birds and this gene was also differently expressed between feather peckers and controls. This suggests that this difference in expression level was due to the different environments in the feather pecking and control cages. The lack of difference between control birds and victims also suggested that the variation in plumage condition did not have any major effects on gene expression. In turn, it may also justify the comparison between feather peckers and victims. Secondly, out of the 16 transcripts that were differently regulated ($p<0.1$) in study IV, 11 (69%) were actually differently expressed when comparing peckers and victims and three

(19%) in peckers compared to both controls and victims. This was not the case in study II, in which only two transcripts were differently expressed in tail biters compared to receivers (corresponding to approximately 1% of the total number of transcripts). This may indicate that the gene expression differences between tail biters and their receivers are fewer than those between feather peckers and their victims.

In conclusion, it is probably always more correct to select the control animals from the same cage/pen as the performer and receiver of an injurious behaviour. But the functions of the differently expressed genes in the feather pecking study are still useful in the exploration of the biological mechanisms underlying feather pecking behaviour.

6 Conclusions

- Pigs that performed tail biting also performed a higher frequency of other abnormal behaviours compared to control pigs. The individuals who performed most tail bites performed other abnormal behaviours that by definition included biting behaviour (bar biting and ear biting) whereas individuals who performed a lower frequency of tail biting performed a variety of other pig-directed abnormal behaviour.

- The high frequency tail biters performed a higher proportion of severe tail bites. The use of severity grading of tail bites based on the reaction of the receiver may help the farmer identify the most frequent tail biters for removal from the pen. It may as well provide opportunities to compare future tail biting studies.

- Neutral pigs housed in tail biting pens, differed in performed and received abnormal behaviour compared to control pigs housed in a control pen. Control pigs *not* housed together with a tail biter performed and received more pig-directed abnormal behaviours (such as belly nosing and ear biting), whereas neutral pigs, that were housed together with at least one tail biter, performed more bar biting.

- A large proportion of the differently expressed genes were differently expressed in neutral pigs compared to tail biters and receivers as well as control pigs. This finding in combination with behavioural differences, led to the hypothesis that neutral pigs have a genetic and behavioural profile that somehow protects them from performing and receiving these behaviours. It is therefore suggested to focus on why some pigs *are not* involved in tail biting behaviour, as opposed to why some pigs *are,* in future studies.

- The functions of the genes that were differently expressed in neutral pigs compared to all other categories, strengthens earlier suggestions that selection for production traits increases tail biting. The gene functions also support the links between tail biting, exploratory and social behaviour.

- When comparing feather peckers with other categories of birds, fewer genes were differently expressed compared to the tail biting studies. Although, a relatively large proportion of these were associated with immune mechanisms, intestinal inflammation and glucose/energy homeostasis. These results support earlier findings that feather pecking is linked to feeding motivation and immune mechanisms.

- Results from earlier studies together with the data presented in this thesis support the similarities between tail biting and feather pecking. Both behaviours are influenced by feeding motivation and are probably affected by intense selection on production traits. Immune mechanisms also seem to be associated with both behaviours.

7 Svensk sammanfattning

När miljön djuren hålls i inte uppfyller deras behov kan de utveckla beteendestörningar, så kallade onormala beteenden. Onormala beteenden förekommer hos många av våra livsmedelsproducerande djur och anses ofta vara ett tecken på försämrad djurvälfärd. Vissa av dessa beteenden riktas mot andra djur i samma miljö, såsom svansbitning hos gris och fjäderplockning hos höns. Onormala beteenden som även skadar andra djur skadas betraktas som extra allvarliga välfärdsproblem. Trots att svansbitning och fjäderplockning påverkas av miljöfaktorer kan det vara svåra att förebygga förekomst av dessa beteenden även i mer djurvänliga produktionsmiljöer. Dessutom är det inte alla djur på en gård som utför dessa beteenden trots att de befinner sig i samma miljö, vilket tyder på att andra faktorer, såsom genetisk bakgrund, kan göra att vissa djur utvecklar dessa beteenden i större utsträckning än andra. För att förstå orsakerna till och öka möjligheterna att förhindra både svansbitning och fjäderplockning, är det viktigt att förstå individuella skillnader mellan djuren och den bakomliggande motivationen till att utföra vissa beteenden. Studierna som beskrivs i den här avhandlingen hade som huvudsyfte att, både genom genetiska och etologiska studier, undersöka biologiska mekanismer som påverkar dessa individuella skillnader med avseende på onormala beteenden.

Ett problem som gör det svårt att jämföra olika svansbitningsstudier är att termen svansbitning kan inkludera allt från mild manipulation av svansen, som inte ger upphov till några skador, till rent kannibalistiskt beteende. Ett av syftena med studie I var att dela in svansbitningsbeteende i tre olika grader beroende på mottagarens reaktion. Mild svansbitning innebar att mottagaren inte reagerade synbart. Svansbitning som graderades som medel innebar att mottagaren gick därifrån eller reagerade med låga ljud och den allvarliga svansbitningen resulterade i att mottagaren vokaliserade högt eller sprang därifrån. Vi kunde visa att grisar som utförde en högre frekvens av svansbitning också utförde en högre andel av den allvarliga formen av svansbitning och en lägre andel av mild svansbitning. Förhållandet mellan de olika svansbitningsgraderna var det motsatta för grisar som utförde en lägre frekvens av svansbitning. Det här kan komma till praktisk nytta för djurägare om de vill identifiera de mest intensiva svansbitarna, eftersom att dessa också får en större reaktion från mottagaren.

Ett annat syfte med studie I var att studera samband mellan svansbitning och andra onormala och oönskade beteenden. Svansbitande grisar delades in i tre olika grupper; högfrekventa svansbitare, lågfrekventa svansbitare och kontroller. Svansbitare utförde mer rörtuggning än kontrollgrisar och dessutom utförde de högfrekventa svansbitarna mer öronbitning än kontrollerna. De lågfrekventa svansbitarna utförde istället mer flanksugning och övriga onormala beteenden jämfört med kontrollerna. Detta resultat indikerar att de grisar som svansbiter även utför andra onormala beteenden som inkluderar någon form av bitbeteende. De lågfrekventa svansbitarna visar istället en större variation inom vilka onormala beteenden de utför.

Ett antal rapporterade studier tyder på att svansbitning till viss del har en genetisk bakgrund, men ännu har inga molekylärgenetiska studier publicerats. Det är inte bara mutationer, dvs. skillnader i DNA sekvensen, som påverkar beteende utan även hur mycket generna uttrycks. I studie II jämfördes genuttryck i hjärnan hos svansbitare, mottagare och neutrala djur (som varken utförde eller utsattes för beteendet). I studie III jämfördes de neutrala djuren även med kontrollgrisar, det vill säga grisar som inte heller utförde eller utsattes för svansbitning. Skillnaden var att de neutrala djuren bodde i en box tillsammans med minst en svansbitare, medan kontrolldjuren bodde i en kontrollbox, där svansbitning inte observerats, i samma byggnad. Detta gjordes med hjälp av genexpressionschip, som kan mäta genuttryck i tusentals gener samtidigt. Flest skillnader i genuttryck fanns hos de neutrala grisarna som, trots att de bodde i samma box som minst en svansbitare, varken utförde eller utsattes för svansbitning. Ett relativt stort antal gener olika uttryckta i de neutrala grisarna jämfört med alla tre övriga kategorier. Detta tyder på att neutrala grisar har en genotyp och fenotyp som gör att de är mindre involverade i svansbitning och att funktionerna av dessa gener kan säga mera om varför vissa djur inte utför eller utsätts för svansbitning.

Bland de gener som var olika uttryckta i de neutrala jämfört med de övriga tre kategorierna fanns gener som i tidigare studier konstaterats påverka produktionsegenskaper hos gris, undersökande beteende hos människa samt socialt beteende hos både möss och människor. Alla dessa tre funktioner skulle alltså kunna ha stor relevans för utvecklandet av svansbitning. Det finns samband mellan svansbitning och vissa produktionsparametrar och man tror att selektion för högre produktion har skapat grisar som är mer benägna att utföra onormala beteenden. Resultaten från beteendestudierna i studie III stärker dessutom att svansbitning påverkas av socialt beteende. I studie III jämfördes frekvensen av andra onormala beteenden mellan neutrala och kontrollgrisar. Kontrolldjuren utförde och utsattes för mer onormala beteenden som var riktade mot andra grisar jämfört med de neutrala grisarna, som istället utförde mer beteenden som var riktade mot inredningen. Detta tyder på att de neutrala djuren skiljer sig från de andra genom att de är mindre fokuserade på social kontakt, vilket leder till att de är mindre involverade i svansbitning.

Vad gäller forskningen om fjäderplockning, så har betydligt fler genetiska studier utförts. Många av dessa har dock utförts på linjer av fåglar som är speciellt selekterade för fjäderplockningsbeteende. Få studier är utförda i kommersiella miljöer. Därför var syftet med studie IV att undersöka genuttryck i hjärnan hos fjäderplockare, mottagare och kontrollhöns på en kommersiell gård som producerar ägg. Färre gener skiljde i uttryck mellan grupper i denna studie jämfört med studie II och III. Dock är funktionerna av några av generna av relevans för fjäderplockningsbeteende. Åtminstone två av generna kan påverka tarminflammationer, vilket är intressant då födosöksbeteende och nutritionsfaktorer är associerat till fjäderplockning. Tarminflammationer kan i längden leda till ett försvagat skelett, vilket också har satts i samband med fjäderplockning. Flera gener med funktioner i olika immunmekanismer var också olika uttryckta hos fjäderplockare jämfört med mottagare och kontroller, vilket också har diskuterats tidigare. I övrigt var också gener med effekt på glukos och energimetabolism olika uttryckta.

Sammanfattningsvis kan vi med dessa studier rapportera listor av gener vars funktioner kan vara viktiga för utvecklingen av onormala beteenden. Många av dessa funktioner var föreslagna som viktiga tidigare, men även nya hypoteser har kommit fram. Bland annat föreslår vi att man undersöker hur selektion för högre produktion påverkar stresshantering, socialt beteende och utvecklingen av beteendestörningar. Tidigare fokus för forskning om onormala beteenden har varit på vilka individuella skillnader som gör vissa djur utför eller mottar svansbitning. Vi föreslår istället att framtida studier bör fokusera på varför vissa djur *inte är* involverade i dessa beteenden till skillnad från de djuren som *är* involverade.

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