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Behaviour of cattle – molecular biological background and relationship to milk performance

Thesis

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To my grandfather

Klaus Hahn

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Abbreviations

ANOVA	analysis of variance
bp	base pairs
BTA	*Bos taurus* autosome
Chr	chromosome
cM	centiMorgan
CNR1	cannabinoid receptor
CYP17A1	steroid-17alpha-hydroxylase
DA	duration of active behaviour
DE	duration of exploratory behaviour
DI	duration of inactive behaviour
DNA	deoxyribonucleic acid
DRD4	dopamine receptor D4
eIF2	Eukaryotic Initiation Factor 2
FC	fold change
FDR	false discovery rate
GWAS	genome-wide association study
h^2	heritability
HPA	hypothalamic-pituitary-adrenal
IPA	Ingenuity® Systems Pathway Analysis
LD	linkage disequilibrium
LSM	Least squares means
Mbp	megabase pair
MY	average daily milk yield (kg)
NCBI	National Center for Biotechnology Information
NH	novel human test
NO	novel-object test
NRF2	nuclear factor erythroid 2-related factor 2
OF	open-field test
PCA	principal component analysis
PC	principal component
PRL-R	prolactin receptor

QTL	quantitative trait loci
RGS2	regulator of G-protein signalling 2
R1	ratio between milk yield one day before and after rehousing
R3	ratio between milk yield three days before and after rehousing
RNA	ribonucleic acid
r_s	correlation coefficient
SAM	sympathomedullary pathway
SD	standard derivation
SE	standard error
SNP	single nucleotide polymorphism
TT	temperament types
TT1	“fearful/neophobic-alert” cows
TT2	“interested-stressed” cows
TT3	“outgoing/neophilic-alert” cows
TT4	“subdued/uninterested-calm” cows
TT5	indistinct cows
TYR	tyrosinase
yield5	milk yield day 1 to 5
yield30	milk yield day 6 to 30

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Chapter 1 General introduction

1.1 Motivation

At the beginning of domestication more than 10,000 years ago, cattle ancestors were among others selected for their adaptability to man-made environments and low reactivity towards humans (Andersson 2001). From about 200 years ago, cattle were primarily selected for their productivity, resulting in a considerable performance increase. As a consequence of selective breeding for production traits, a shift of behavioural characteristics in *Bos taurus* species can be observed due to modifications on the individual reactivity threshold, resulting in quantitative rather than qualitative differences (Mignon-Grasteau *et al.* 2005). Today, there is again increasing interest on the selection for cattle behaviour in dairy and beef breeds (Haskell *et al.* 2014). Behavioural characteristics, in cattle often referred to as temperament, describe stable behavioural and physiological response to stressors or challenging situations and are said to have a profound effect on livestock production. Behavioural characteristics like stress responsiveness or calmness, were shown to be associated with economically important production traits, such as milk yield in dairy cattle (Breuer *et al.* 2000; Hemsworth *et al.* 2000; Sutherland *et al.* 2012) as well as with meat quality and the average daily weight gain in beef cattle (Voisinet *et al.* 1997; Petherick *et al.* 2002; King *et al.* 2006; Vetters *et al.* 2013). Moreover, the ability to adapt to different environments determines the individual stress responsiveness and thus affects animal welfare (Jensen *et al.* 2008). Finally, docility and manageability, especially in dairy cattle or intensively housed beef cattle, can influence longevity because behaviour can be used as culling or selection criteria.

The hypothesis of a genetic background of behaviour is strongly supported by empirical evidence, but precise information on molecular and physiological mechanisms underlying cattle behaviour are still limited. Especially in cattle, the topic of behaviour genetics is merely addressed in comparison to rodents or other livestock species (Adamczyk *et al.* 2013). In recent studies, a genetic background of cattle behaviour has been indicated due to observed breed differences (Gauly *et al.* 2002), a moderate to high heritability (h^2 = 0.13 to 0.51) for temperament traits (Sewalem *et al.* 2011; Riley *et al.* 2014) and mapped quantitative trait loci (QTL) for behaviour-related traits (Hiendleder *et al.* 2003; Gutiérrez-Gil *et al.* 2008). Moreover, changing concentrations of the stress hormones cortisol and epinephrine associated with cattle temperament in challenging

situations indicate potential physiological mechanisms underlying behaviour (Cafe *et al.* 2011a; Burdick *et al.* 2011b) and highlight the role of the adrenal cortex as possible target tissue.

Information on the genetic and physiological relationship between cattle behaviour and production traits is rare and limited to beef production traits. For example Lindholm-Perry *et al.* (2015) performed a genome-wide association study (GWAS) for cattle temperament and revealed a positive relationship for genotype effects on superior feed efficiency (higher average daily weight gain and feed intake) and desirable temperament (low flight speed) for genetic markers on BTA6. A phenotypic correlation between milk performance and behaviour-related traits, such as a loss in milk yield as reaction towards handling or novelty, was described in other studies (Breuer *et al.* 2000; Sutherland *et al.* 2012). Furthermore, several QTL for milking temperament, a trait which can be seen as phenotypic combination between behaviour and milk performance, were identified (Schrooten *et al.* 2000; Schmutz *et al.* 2001; Hiendleder *et al.* 2003). Thus, a molecular relationship between cattle temperament and milk performance can be expected and therefore should be analysed.

This thesis aims to provide information on the biological background of cattle behaviour using different molecular biological approaches in a F2 Charolais x Holstein cross breed. In addition, first investigations of the biological relationship between cattle temperament and milk performance, as a major production trait, were performed.

1.2 Behavioural characteristics in cattle

Terminology for behaviour is not consistently used in literature and depends on the field of research and investigated species. A behavioural trait which is shared by the members of a population and that vary among individuals is considered as characteristic. The characteristic style of an individual's behaviour responsiveness in different situations describes the temperament, which can comprise the categories shyness-boldness, exploration-avoidance, activity, sociability and aggressiveness (Réale *et al.* 2007). In livestock animals, behavioural characteristics are usually evaluated with respect to handling. Thus, frequent cited definitions for cattle temperament similarly

refer to the same underlying concept. For instance Burrow (1997) referred to cattle temperament as "*the animal`s behavioral response to handling by humans*". In a more general definition, Sutherland *et al.* (2012) described cattle temperament as "*consistent behavioral and physiological differences observed between individuals in response to a stressor or environmental challenge*". The concept of stable behavioural characteristics over time and across situations sums up the current view on temperament (Réale *et al.* 2007; Koolhaas *et al.* 2010). Results in cattle research provided supporting evidence for behavioural consistency. Müller & Schrader (2005) observed a high repeatability in measured behavioural traits, for example, exploration, walking and vocalization, in response to social isolation throughout two lactations in dairy cattle. In coincidence, a long-term intra-individual consistency was shown for locomotion and vocalization during an open-field test from rearing to first pregnancy or first lactation (van Reenen *et al.* 2013). Moreover, a long-term relationship between stress-responsiveness as behavioural characteristic, and physiological parameters was reported by Terlouw *et al.* (2012). In their study, indicators of post mortem muscle metabolism reflected the individual stress-reactivity at slaughter. Interestingly, these indicators were additionally correlated to behavioural reactions and heart rate variability in earlier reactivity tests.

The observed interactions between behavioural characteristics and physiological parameters give initial indications for the mechanisms underlying the effect of cattle temperament on productivity and welfare. Both, in dairy and beef breeds, more favorable behavior, like calmness, was positively associated with an increase of important parameters of production. In this context, the physiological stress response seems to play a significant role. In dairy cattle, increased stress-responsiveness towards human handling or novelty was a limiting factor for milk yield (Hemsworth *et al.* 2000; Breuer *et al.* 2000; Sutherland *et al.* 2012). As a key mechanism behind this observation, the inhibition of oxytocin release, a hormone responsible for milk let-down, due to stress, is discussed (Bruckmaier & Blum 1998). Another possible explanation is the suppression of oxytocin effects, since Sutherland *et al.* (2012) observed increased oxytocin levels, but a drop in milk yield when cows were milked in novel environments in comparison to measurements when milked in the familiar milking parlor.

1.3 Biological architecture of cattle behaviour

Genetics and behaviour

The characteristics of behaviour are not only attributable to environmental influences, experiences and sex, rather there is an interaction of external stimuli and internal factors. To show a specific behaviour, physiological structures are required to perceive external stimuli (sensory organs), transmit and process the information (nervous system, brain) and induce a reaction (flight vs. fight) via the neuromuscular or neuroendocrine systems (Baker *et al.* 2001). Thus, genes, which are the basis of all physiological processes, are assumed to be involved in behavioural variations. In line with the thesis of a genetic paradigm of behaviour, several genomic regions were identified to harbour deoxyribonucleic acid (DNA) variations significantly associated with behaviour in cattle in QTL mapping studies using microsatellite markers. These QTL were distributed over the whole bovine genome (Schmutz *et al.* 2001; Hiendleder *et al.* 2003; Gutiérrez-Gil *et al.* 2008; Glenske *et al.* 2011). In contrast to other species, where candidate genes were successfully identified (Bendesky & Bargmann 2011), no strong indications for candidate genes have existed in cattle until now (Lühken *et al.* 2010; Glenske *et al.* 2011). Overall, behaviour is considered to be a polygenic trait, i.e. small effects of numerous genetic loci were accumulated to shape overall behaviour (Jensen 2006). It was additionally shown that genetic factors cause only small proportions of the phenotypic variability in relation to the proportion of environmental factors (Flint 2003).

Physiological mechanisms and behaviour

A scheme for all processes, structures and stimuli that contribute to the behavioural phenotype is illustrated in Figure 1.1. The effect of genetic variances on behaviour is not direct; rather the impact is manifested at other levels, including transcripts, proteins, metabolites and finally complex networks of neurophysiological and structural factors (Johnston & Edwards 2002). Especially substances which act as neurotransmitters are critical in the determination of behavior. Thus, the serotonergic and catecholaminergic systems, and their interaction, were focused in research for synthesis and metabolism of pivotal neurotransmitters, for example, serotonin and dopamine (Mormède 2005). The catechol-O-methyltransferase was recently shown to affect dopamine synthesis (Dang *et al.* 2013). Dopamine, in turn, was associated with numerous behavioural characteristics

in different species, for example, novelty seeking and curiosity (Bailey *et al.* 2007; Munafò *et al.* 2008; Korsten *et al.* 2010) or memory (Shohamy & Adcock 2010). Shohamy & Adcock (2010) introduced the concept of "adaptive memory": Memory mediated experiences are crucial for adaptive behavior in novel situations. They reviewed that dopamine plays a central role in "adaptive memory", because this neurotransmitter emerged to modulate the hippocampus, an important brain area underlying long-term memory. Moreover, the histaminergic system was shown to affect behavioural characteristics like locomotor activity, memory, cognition, anxiety and obesity in different types of knock-out mice (Schneider *et al.* 2014) due to the role of histamine as a neurotransmitter.

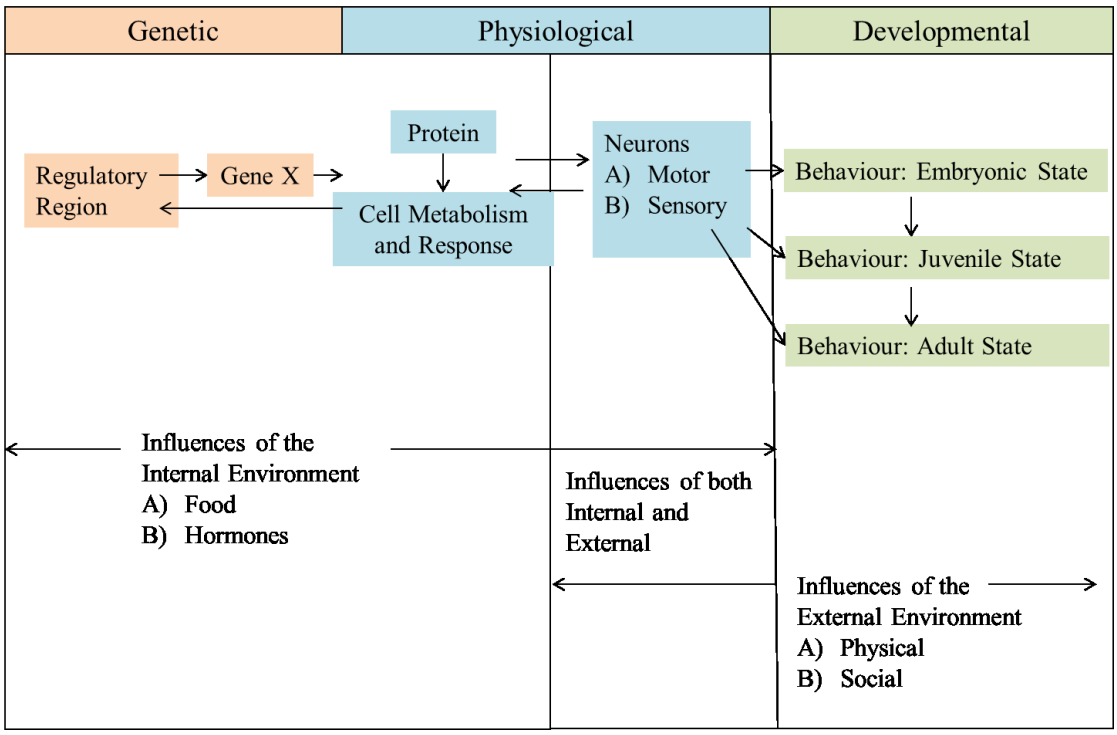

Figure 1.1 An integrative model for the short-term causes of behaviour in the life of an animal adapted from Hager (2010)

In cattle, little is known about endocrine regulation with regard to behaviour. Results at the physiological level were observed for the stress reactivity in response to handling or novel situations, where the hypothalamic-pituitary-adrenal (HPA) axis and sympathomedullary pathway (SAM) were involved (King *et al.* 2006; Curley Jr. *et al.*

2008; Cafe *et al.* 2011a; Burdick *et al.* 2011b). More excitable cattle were observed to have higher basal concentrations of glucocorticoids (for example cortisol) and catecholamine hormones (for example epinephrine) in stressful situations in comparison to calm cattle (Curley Jr. *et al.* 2008; Burdick *et al.* 2011a). Other concrete investigations into the biological mechanisms behind behavioural traits in cattle were done looking specifically at estrous behaviour due to its impact on insemination and thus, on fertility (Boer *et al.* 2010; Kommadath *et al.* 2011). In these studies, effects of sexual hormones, for example, estradiol and progesterone, and the gonadotropin releasing hormone were primarily involved in variances in estrous behaviour. Moreover, the altered expression of genes coding for oxytocin and arginine vasopressin could be connected to different states of estrous behavior (Kommadath *et al.* 2011).

1.4 Analysing the biological background of cattle behaviour

Strategies for behaviour phenotyping

Understanding the biological background of cattle behaviour is a relatively new field of research and has several difficulties: "*Behavioural traits are difficult to study because of the strong environmental component and because it is difficult to collect objective and informative records, in particular on the number of animals needed for high resolution mapping. However, behavioural genetics in domestic animals is an exciting field for future research* (…)" (Andersson 2001). To minimize these limitations, the analysis of an appropriate experimental population characterized by a high individual phenotypic variability is of particular importance. Domestic animals, like cattle, represent powerful systems in the genomic dissection of complex traits. In the genetic analysis of quantitative traits, the use of segregating populations, i.e. experimental crosses, is well established (Andersson & Georges 2004). According to the principles of the genetic theory of Mendelian inheritance, intercrossing the offsprings of genetically and phenotypically divergent individuals resulted in informative resource populations [F2 design] (Kogelman *et al.* 2013). Thus, a F2 design represents an important prerequisite in the genomic and biological exploration of complex traits.

In 2003, a F2 population of a cross between German Holstein cows and Charolais bulls was established at the Leibniz Institute for Farm Animal Biology (FBN) Dummerstorf

to analyse the different types of nutrition turnover (SEGFAM; Kühn *et al.* 2002). Both, German Holsteins and Charolais, represent typical dairy and beef cattle breeds, respectively, with low intra and high inter breed phenotypic variability due to different breeding purposes. During handling procedures that were necessary within the scope of the study of Kühn *et al.* (2002), for example blood sampling, cows showed a high variability regarding approachability and excitability. Accordingly, distinct differences between dairy and beef cattle were reported, indicating dairy cattle to be less reactive towards handling but more reactive to sudden events in comparison to beef breeds (Murphey *et al.* 1980; Lanier *et al.* 2000). To gain a deeper insight into the SEGFAM behavioural phenotype, the animal`s behaviour was investigated in a previous study in the context of behavioural biology. In the study conducted by Graunke *et al.* (2013), numerous behavioural traits were recorded in a novel-object test to describe cattle temperament in a multidimensional manner using a principal component analysis. As a result, two principal components, novel-object-related and exploration-activity-related, were identified that explained up to 58% of the corresponding behavioural trait variability. Additionally, considerable individual differences were observed in milk performance for the F2 SEGFAM cows (Hammon *et al.* 2007). Lactation curves showed distinctive deviations from usual lactation duration and persistence, especially within 30 days postpartum, milk yield was low.

A main problem in the analysis of cattle temperament is the assessment of suitable behavioural phenotypes. Phenotyping cattle behaviour should be less time and space consuming, safe for the handler and as informative as possible. In beef cattle, behavioural scoring is usually conducted during routine handling procedures. For instance during fixation in a squeeze chute or during weighting, numbers of movements or the exit flight speed were measured to determine excitability (Benhajali *et al.* 2010; Black *et al.* 2013; Vetters *et al.* 2013). In dairy cattle, routine behavioural assessments are less common. However, a subjective behavioural assessment can be done during milking. This can be referred to as milking temperament or milkability (Schmutz *et al.* 2001; Hiendleder *et al.* 2003).

Another strategy is the application of standardized test situations, which are often adapted from behavioural research in laboratory animals, for example an open-field (OF) or novel-object (NO) test. Although behavioural tests are more extensive regarding

time, space and work, they could be particularly insightful within the field of cattle behaviour for several reasons. It is possible to record multiple behavioural traits that can be specifically selected with respect to the behavioural characteristic of interest (Réale *et al.* 2007). Moreover, disturbing influences can be limited to a greater extent in comparison to routine handling procedures and experiences with model organisms can be used. An open-field test for the assessments of dairy cattle temperament was introduced by Kilgour (1975). The advantages of this test are the easy construction and the creation of a completely new environment that allows for the testing of numerous behaviour characteristics, like animal's reactivity towards unfamiliar environments and social separation. When an unknown object is added, referred to as novel-object test, the test situation primarily addresses the behavioural characteristics 'fear' and 'curiosity/novelty seeking' (Réale *et al.* 2007). Finally, the ability to cope with human handling and presence are key psychological aspects of cattle temperament in general livestock practice (Adamczyk *et al.* 2013). Involving humans in behavioural test was reported to support fearfulness as the main component underlying cattle behaviour (Mazurek *et al.* 2011).

Application of 'omics' technologies

Behaviour is considered a multifactorial trait that is strongly influenced by the environment and analyzing the underlying biological mechanisms of phenotype expression in a comprehensive manner can provide valuable information. The term "system biology" refers to the simultaneous study of high throughput "omics"-data including the genome, transcriptome, proteome and metabolome of an organ, tissue, or an organism at different condition. This is carried out using state-of-the-art statistical or quantitative genetic, computational biology and bioinformatic principles and tools (Romero *et al.* 2006; Kadarmideen 2008). Figure 1.2 highlights recent applications at different 'omics' levels for a holistic analysis of numerous phenotypes and biological questions and challenges. Investigations of high-throughput data at different 'omics'-levels were successfully applied to reveal complex processes and issues, i.e. breast cancer in humans (Wang *et al.* 2014) or puberty in beef cattle (Cánovas *et al.* 2014).

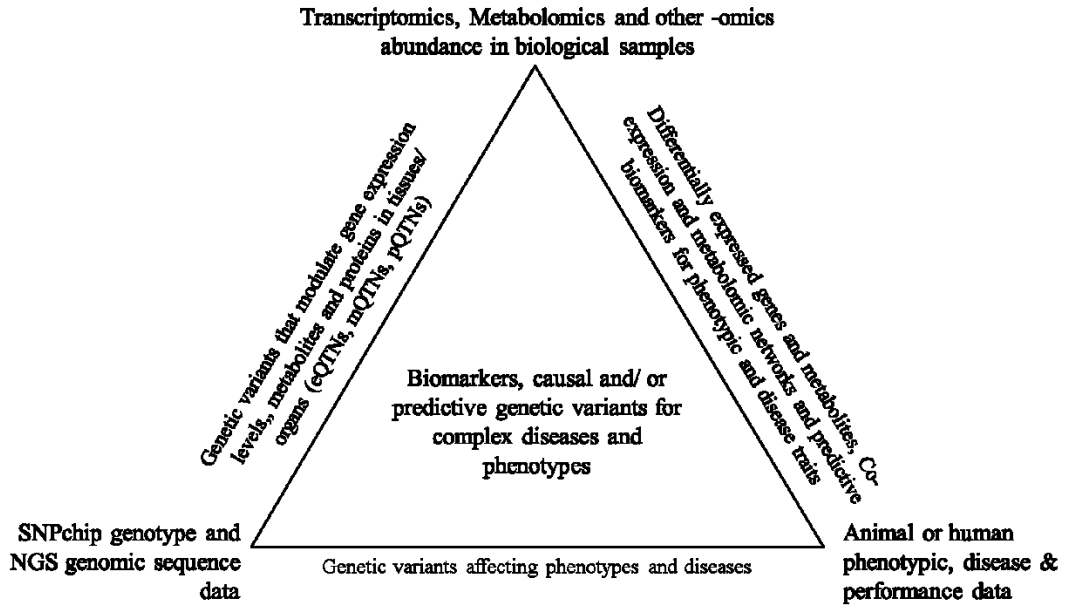

Figure 1.2 Schematic representation for the integration of 'omics data' in biological analyses adapted from Kadarmideen (2014)

In livestock animals, genomics (for example GWAS) and functional genomics (for example transcriptomics) based on high-density SNP arrays and microarrays are commonly used in the context of complex biological questions and for the progress of genomic selection. GWAS are based on the phenomenon of population-wide linkage disequilibrium (LD) between single-nucleotide polymorphism (SNPs) as genetic markers and causal variants (Kadarmideen 2014). A SNP is a DNA variation where a single base is deleted, inserted or substituted by another single base. SNPs are highly abundant throughout the whole genome of individuals and could enormously contribute to our understanding of the relationship between genetic variation and biological function (Syvänen 2001). When an association analysis between high-density SNP genotype data and an observed phenotype is performed, several statistical problems can occur. First, in the case of testing multiple SNPs, an adjustment of the significance threshold is necessary to decrease the number of false-positives due to multiple testing, which is commonly done by using Bonferroni correction or based on false discovery rate (FDR) adjustment (Sham & Purcell 2014). Second, a common problem in GWAS is the existence of hidden population stratification. Population stratification describes systematic ancestry differences which can lead to subgroups in a population with differences in allele frequency and LD (Price *et al.* 2010). An approach to address these

issues is the use of mixed models, where SNP effects were estimated while simultaneously fitting family polygenic effects to correct stratifications (Kadarmideen 2014).
Up to now, the use of high-throughput SNP analyses, for example in GWAS, was primarily limited to breeding for performance traits or disease resistance in livestock species (Kadarmideen 2014), but may result in valuable insights in the context of cattle behaviour.

Transcriptome studies aim to deduce and quantify the complete set of ribonucleic acid (RNA) in a cell for a specific developmental stage or physiological condition using microarray or RNA sequencing technology in order to identify differentially expressed genes (Wang *et al.* 2009). Microarray experiments can enable the joint analysis of up to 30,000 transcripts (Kadarmideen 2014) which complicates the statistical analysis due to a large number of variables (Loor *et al.* 2013). Usually, univariate approaches were implemented for the detection of expression differences between tissues, treatments, groups or time points, for example analysis of variance (ANOVA) and cluster analysis. To determine the relationship between continuous data sets, methods like the Spearman rank correlation were commonly used. In cattle, transcriptomics were mainly conducted to analyse different physiological states and conditions of lactation (Loor 2010; Bionaz *et al.* 2012; Loor *et al.* 2013) or compare alterations in expression profiles associated with reproduction (Evans *et al.* 2008) and diseases, for example mastitis, bovine spastic paresis or transmissible spongiform encephalopathies (Khaniya *et al.* 2009; Pariset *et al.* 2013; Sasaki *et al.* 2014). In the context of cattle behaviour, microarray analysis was used to study estrous behaviour (Kommadath *et al.* 2011), but research on overall temperament is missing.

1.5 Objectives and structure of the thesis

The primary goal of this study was to explore the underlying biological mechanisms of cattle behaviour in the SEGFAM F2 resource population at the genome and transcriptome level. Further, this work is intended to offer new insights into the relationship between cattle temperament and milk performance.

In a literature review, current knowledge on the topic of cattle temperament and genetics as well as open questions and thus, further fields of research, were summarized (Chapter 2). This review highlights the importance of cattle temperament in conventional production systems and the influence on production traits. Further, the question of a biological background of cattle behaviour was addressed by investigating what is known on this subject in general and particularly in cattle. Finally, common problems with behavioural phenotyping and the selection of cattle temperament were stressed.

SNPs were associated to behavioural traits in a GWAS (Chapter 3) to gain further insights into the genetic background of cattle behaviour and to identify molecular markers that might help to discriminate behavioural traits at the genetic level. Further, genotype effects on behaviour and milk performance have not been addressed before using SNP data and thus, SNPs significant for behavioural traits have been analysed regarding their effect on different milk performance traits in this thesis. Finally, a drop in milk yield was observed for some cows after rehousing during the first days of lactation. This observation was quantified with a ratio analysed as a combined trait for milk performance and behaviour to evaluate the effect of novelty on milk performance.

A physiological mechanism that was shown to have an impact on stress reactivity in livestock species is the HPA axis. To examine the role of the adrenal cortex in the determination of cattle temperament, the bovine adrenocortical transcriptome was analysed after slaughtering, which subjects the animals to emotional stress due to novelty, social isolation and handling (Chapter 4). To evaluate the relationship between adrenocortical gene expression and behavioural characteristics over time, the expression profiles were associated with temperament types that the cows were assigned to in a novel-human (NH) test early in age. In both, slaughtering and NH testing, humans were

involved. Human handling was reported to be a main stressor underlying cattle temperament and thus, the significantly different transcripts between temperament types were functionally analysed, especially in regard to pathways of the physiological stress response.

Chapter 2 Genetics of cattle temperament and its impact on livestock production and breeding– a review

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Genetics of cattle temperament and its impact on livestock production and breeding– a review

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Abstract

Cattle temperament, which describes individual behaviour differences with regard to a stressor or environmental challenges, is known for its impact on working safety, adaptability to new housing conditions, animal performance and for evaluation of animal welfare. However, successful use of temperament in animal breeding and husbandry to improve keeping conditions in general or animal welfare in particular, requires the availability of informative and reproducible phenotypes and knowledge about the genetic modulation of these traits. However, the knowledge about genetic influences on cattle temperament is still limited. In this review, an outline is given for the interdependence between production systems and temperament as well as for the phenotyping of cattle temperament based on both behaviour tests and observations of behaviour under production conditions. In addition, the use of temperament as a selection criterion is discussed.

Introduction

During the last several decades, new management systems have been introduced worldwide in cattle production, presenting new challenges for animals and farmers. In particular, the increasing automation of routine processes and growing herd sizes due to the intensification of livestock production limit the contact between cow and farmer (Raussi 2003) and contributes to fear of humans and stressful events (Boissy *et al.*

2005). Since the ability of cattle to cope with external stimuli affects the susceptibility to stress (Jensen 2006), stress from routine management processes, like the regrouping of a herd, can result in aggressiveness, increased locomotion and decreased productivity if coping strategies are insufficient (Bøe & Færevik 2003). Increased stress has additionally been shown to affect physiological processes of the immune and reproductive system negatively (Burdick *et al.* 2011b). Accordingly, cattle temperament, which describes "*consistent behavioural and physiological differences observed between individuals in response to a stressor or environmental challenge*" (Sutherland *et al.* 2012) is found to have a considerable impact on performance, reproduction, health and animal welfare. Temperament comprises behavioural characteristics like shyness-boldness, exploration-avoidance, activity, sociability and aggressiveness and is an important aspect of behaviour genetics (Réale *et al.* 2007).
Based on the theory that animal welfare comprises the animals' "*state as regards its attempts to cope with its environment*" (Broom 1986) and with the evaluation of emotionally positive surroundings (Veissier *et al.* 2012), the selection for temperament types that are well suited for specific production systems is expected to improve productivity and overall animal welfare (Boissy *et al.* 2005; Ferguson & Warner 2008). Animal welfare covers the physiological state, biological needs and furthermore the emotional condition of animals (von Keyserlingk *et al.* 2009). Criteria for the evaluation of animal welfare were introduced years ago by the concepts of the Farm Animal Welfare Council (FAWC 1979) and by Bartussek *et al.* (2000), but in spite of different approaches, it is complicated to assess the emotional state of cattle since these concepts are mainly based on environmental factors. However, a novel approach, the Animal Welfare Assessment Protocol, introduced animal-based measurements including behaviour for assessing animal welfare (Welfare Quality® 2009). The assessment of cattle behaviour in certain situations could provide additional information on the physiological and emotional state of the animal overall, improving animal welfare evaluation.

Besides environmental influences, genetic factors are known to contribute to the development of the behaviour phenotype (Mormède 2005). The possible genetic predisposition of temperament and the potential impact of temperament on cattle welfare and production traits has focused attention on behavioural phenotyping and the opportunity of selection for temperament.

However, integrating cattle temperament in breeding programs is difficult. Temperament is assumed to be multidimensional, and due to the complexity of behavioural traits there is no single objective measurement that is able to capture all behavioural characteristics (Réale *et al.* 2007). In addition, Oltenacu & Broom (2010) found a conceivable competitive relationship between the genetic selection for dairy production and adaptability due to limited physiological resources, resulting in poorer adaptability by selection for milk yields. Furthermore, Grandin (1994) discussed that the masking of unfavourable behavioural traits like nervousness, flightiness or excitability by adaption to the human-created environment of livestock production hinders the selection for behavioural traits like temperament. One possibility for overcoming these problems is the analysis of the genetic background of cattle behaviour, which could contribute to the successful integration of temperament in breeding programs by the use of temperament associated markers (marker-assisted selection or genomic selection) and further help to evaluate the correlation between temperament and performance. The most important prerequisite to identify genetic loci affecting temperament is the development of distinct informative and reproducible phenotypes characterizing different temperament types.

Phenotyping cattle temperament

Cattle temperament and production systems

Particular experiences, especially early ones, are important in the development of temperament in cattle. On average, young cattle were observed to be more temperamental than older cattle (Voisinet *et al.* 1997; Lanier *et al.* 2000) and with aging, cattle' behaviour was found to be more consistent over time (Gibbons *et al.* 2011; Haskell *et al.* 2012). These modulations of behaviour through individual experiences and therefore through aging are assumed to evolve from changes in the reactivity of the nervous system (Grandin and Dessing 1998). The graduate adaption to repeated external stimuli is referred to as habituation (Cyr & Romero 2009). In livestock production, habituation is mainly determined by the adaptability to human-made environments and the frequency of human-animal contact overall, depending on the production system. Extensively kept cattle, for example, are occasionally handled and are therefore less approachable than intensively housed beef or dairy cattle (Le Neindre *et al.* 1996). As a

consequence of the adaption and selection for different production and housing systems, a large variability in temperament exists today in farm animals, resulting from differences in reactions towards human contact and new surroundings (Hopster 1998; Sutherland *et al.* 2012). Fear is considered one of the main psychological factors underlying temperament traits in general, and in particular, fear of humans affects the human-animal relationship considerably (Adamczyk *et al.* 2013). When humans were involved in behaviour tests it could be observed that fearfulness was more evident in comparison to tests without human presence (Mazurek *et al.* 2011). The degree of fearfulness, or avoidance, of humans is indicated by the flight distance or flight speed that is known to depend on the frequency and quality of human-cattle habituation (Waiblinger *et al.* 2003; Schütz *et al.* 2012) and can be measured when an animal flees to avoid human contact. Besides individual experiences and aging, the influence of sex on cattle temperament is discussed. Some beef cattle studies documented that cows had higher temperament scores than steers (Voisinet *et al.* 1997; Gauly *et al.* 2002; Hoppe *et al.* 2010). Just as the production system promotes certain behavioural characteristics, animal-specific temperament can likewise affect relevant parameters in livestock production. Docility in cows, for example, was observed to affect reproduction traits positively, including the calving rate, the age at first observed oestrus (Phocas *et al.* 2006) and conception rates (Cooke *et al.* 2011). Furthermore, a negative correlation was reported between fear of humans and milk yield (Hemsworth *et al.* 2000) explaining up to 19% of the milk yield variances between farms observed in the study of Breuer *et al.* (2000). The dynamics of the hormone oxytocin have been widely analysed as a possible explanation for the correlation between temperament and milk performance. Bruckmaier & Blum (1998) summed up that the release of oxytocin may be repressed by the central nervous system due to increased levels of β-endorphin and cortisol when cows were milked in novel environments. Rushen *et al.* (2001) documented lower plasma oxytocin concentrations in unfamiliar milking parlours confirming a negative effect of novelty on milk production, whereas Sutherland *et al.* (2012) found higher oxytocin concentrations and a drop in milk yield after milking in novel environments. They discussed variations in the activation of the sympathetic nervous system as causal physiological mechanisms for disturbances in milk letdown by peripheral inhibition of oxytocin effects, as it is suggested by Van Reenen *et al.* (2002). In a study of Orbán *et al.* (2011), no correlation between milk yield and temperament could be detected, but calmer cattle had lower somatic cell counts.

In beef cattle, negative side effects of temperament on the average daily weight gain, live weight and meat quality were reported in various studies (Voisinet *et al.* 1997; Gauly *et al.* 2001; Petherick *et al.* 2002; King *et al.* 2006; Nkrumah *et al.* 2007; Hall *et al.* 2011; Vetters *et al.* 2013). In *Bos taurus* steers, for example, docility resulted in up to 0.19 kg higher average daily weight gains (Voisinet *et al.* 1997). The individual temperament is discussed to affect weight gains through influencing the feed conversion efficiency (Petherick *et al.* 2002) and inducing differences in feed intake and time spent eating (Cafe *et al.* 2011b). In addition differences in the susceptibility to stress during slaughter were shown to result in variances regarding meat quality. Calm animals were observed to have significantly higher postmortem pH values (King *et al.* 2006) and more tender meat (Hall *et al.* 2011). Magolski *et al.* (2013) tried to explain the mechanisms behind the correlation of temperament and beef tenderness by analysing the association between protein degradation, calpain system activity and temperament but no significant explanatory relationship could be identified. Despite more and more studies on a possible correlation between cattle behaviour and production traits, inconsistent findings illustrate the demand for further research and standardized tests to elucidate the underlying mechanisms.

Measuring the behavioural phenotype in cattle

In cattle, many approaches exist for measuring behaviour. A detailed overview about different behaviour test conditions and their use in farm animals is given by Canario *et al.* (2013). Behaviour tests are often adapted from behavioural studies of laboratory rodents and can be distinguished based on the type of test (restrained or non-restrained), the data assessment (during routine handling or specific test conditions) and the type of measured trait (qualitative or quantitative). One example is the open-field test, which is well documented and frequently used in model animals. The open-field test can be classified as non-restrained test where the cow is free to move within a defined testing area. Kilgour (1975) introduced the open-field test for the assessment of temperament in dairy cows for its several advantages which are simple construction and the creation of a completely new environment, allowing the testing of numerous behavioural characteristics, like reactivity towards novelty and social isolation. Critical aspects of behaviour assessments in artificial test situations are the time and space requirements to conduct the behaviour test. Therefore behaviour is commonly evaluated during routine handling processes since they are not highly time and space consuming. In dairy cattle,

for example, behavioural assessment is usually conducted by scoring temperament for nervousness, aggressiveness or docility during milking by farmers or milking technicians (Dickson *et al.* 1970; Hiendleder *et al.* 2003). However, in beef cattle, scoring during weighing is a frequent test for determining temperament. When cattle`s opportunities to move are limited, as in a chute during weighing and milking, this is referred to as a restrained test (Burrow 1997), the main advantage of which is safe application for the handler (Boivin *et al.* 1992). A restraint test is able to quantify characteristics like the chute score or flight speed (exit velocity) to evaluate the temperament in response to a short time fixation (Black *et al.* 2013; Vetters *et al.* 2013; Magolski *et al.* 2013). During fixation, the number of movements is suggested to be as most promising trait for selection of beef cattle temperament in Benhajali et al. (2010). In their study, the number of movements during weighing had the highest heritability ($h^2 = 0.31 \pm 0.10$), with a high number of steps implying more agitated animals.

Also challenging, but essential for investigating behaviour in cattle, is the interpretation of behavioural traits which are usually expressed by only a few animals (Broucek *et al.* 2008). Such traits, like vocalization or escape events out of the testing area, are highly informative but complicate statistical evaluation. The determination of the behaviour phenotype can be done qualitatively by temperament scoring or quantitatively by measurements of objective parameters like time spent running, number of escapes, flight time or vocalization events (Watts *et al.* 2001; Gutiérrez-Gil *et al.* 2008; Cafe *et al.* 2011b). In general, the use of automatic measurement integrated into routine processes, for example weighing or milking, is desired in the determination of cattle temperament with regard to time-management and objectivity. In various studies, it could be shown that the determination of behavioural traits or temperament was successful using automated detection. König *et al.* (2006) recorded the frequency of voluntary entries into an automated milking system in dairy cows and proposed this trait as breeding criterion for cattle behaviour and Schwartzkopf-Genswein *et al.* (2012) suggested two electronic measuring systems for the prediction of cattle temperament. In their study, the assessments of strain gauges and accelerometers for the movements of cattle in a squeeze chute were highly correlated to subjective temperament scores.

Depending on the procedure of behaviour assessment, specific behaviours are stimulated, for example, exploratory behaviour in an open-field test or fear of humans in human-approach tests (Réale *et al.* 2007). This specificity hinders the comparability

between different testing conditions as it was shown for a human approachability and novel stimuli tests in Gibbons *et al.* (2009). Although temperament scoring is subjectively due to the perception of the observer, but usually based on experimental protocols, it could be shown that temperament scores were favourably correlated to quantitative records (Schwartzkopf-Genswein *et al.* 2012). For increasing the accuracy of the determined phenotype or temperament type, the combination of behaviour records and physiological and endocrinological parameters are used in behaviour studies. Measurements of cortisol and heart rate are often used to measure the activity of the hypothalamic-pituitary-adrenal axis and sympatho-adrenal medullary system as supplementary indicators for the stress response in cattle (Grignard *et al.* 2001; King *et al.* 2006; Curley Jr. *et al.* 2008; Burdick *et al.* 2010; Cafe *et al.* 2011a). Higher heart rates and cortisol levels indicate more excitable or temperamental cattle. Furthermore, Burdick *et al.* (2010) found a positive correlation between temperament and rectal temperature. A rather rarely applied approach for evaluating behaviour in cattle was used in the study of Core *et al.* (2009). They found a highly significant correlation, ranging from 0.67 to 0.95, between the eye-white percentage and temperament scores assessed in a chute test in beef cattle. Besides the analysis of behavioural traits and physiological parameters, the additional consideration of genetic information could help to discriminate between behaviour phenotypes and reveal differences and commonalities between the particular applied test conditions and measured behaviours.

Genetic variances affecting temperament in cattle

Genetic background of cattle temperament

Today, the genetic background of cattle temperament is generally accepted. A first indication for a genetic predisposition and an essential process leading to the development of the contemporary livestock behaviour is found in the domestication of cattle ancestors beginning 10,500 years ago. At that time, animals were selected for their adaptability to man-made environments and their reactivity towards humans. Therefore, tameness and adaptability can be seen as main fitness-determining factors (Price 1999) which are assumed to be under genetic control (Baker *et al.* 2001). Further evidence for a genetic predisposition of cattle behaviour are in the observed variances in inter-breed temperament. These differences can be attributed to the selection for specific

production systems as well as housing and climatic conditions. In general, *Bos indicus* breeds were found to be more excitable than *Bos taurus* breeds (Voisinet *et al.* 1997). Dairy cattle showed a higher approachability than beef cattle (Murphey *et al.* 1980) and were more reactive to sudden noises during cattle auctions (Lanier *et al.* 2000). Moreover, numerous behaviour studies were conducted for different beef breeds enabling a temperament ranking from more calm breeds like Herford and Angus to breeds that are more temperamental like German Simmental or Charolais (Morris *et al.* 1994; Gauly *et al.* 2002; Hoppe *et al.* 2010).

Estimated heritabilities for temperament, which are rather low or moderate, indicate a lower proportion of a genetic predisposition on the phenotypic variance. In Holstein cows, early estimates for milking temperament ranged from 0.11 to 0.17 (Lawstuen *et al.* 1988; Visscher & Goddard 1995; Rupp & Boichard 1999; Schrooten *et al.* 2000). In a more recent study, heritability reached values of 0.13 and 0.25 for milking temperament and milking speed in Canadian Holstein cattle (Sewalem *et al.* 2011). The estimated heritability for temperament traits in beef cattle is on average higher but with a greater margin, ranging from 0.11 to 0.61, presumably due to different behaviour phenotypes and sample sizes (Burrow 2001; Phocas *et al.* 2006; Nkrumah *et al.* 2007; Hoppe *et al.* 2010). Besides the acceptance of genetic variances contributing to the modulation of behaviour, current knowledge about genotype-phenotype interactions is still limited. One reason, the complexity of behavioural traits, has been discussed; the complexity is often distinguished by different genetic loci and therefore expected to be polygenic traits with quantitative inheritance patterns (Jensen 2006).

The genetic impact on behaviour is not direct, but results from a complex response network of neurophysiological and structural factors, like hormones and proteins, themselves products of indirect genetic effects (Johnston & Edwards 2002). It is assumed that proteins involved in this process have rather general functions, like protein kinases (Price 2008). Protein kinase C, for example, was recently identified as a regulator of mood-related behaviours in rats (Abrial *et al.* 2013) and protein kinase G is discussed to affect diverse behaviours in different species (reviewed in Reaume & Sokolowski 2009). Important neurotransmitters that contribute to the development of behaviour are assumed to arise from the serotonergic or catecholaminergic system (Mormède 2005). A frequently investigated physiological pathway with a high inter-

individual variability that can modulate behavioural characteristics is the stress response mediated through the HPA axis. HPA axis activity and aggressive behaviour were recently reported to be associated with two single nucleotide polymorphisms (SNPs) in pigs (Muráni *et al.* 2010). Likewise in cattle, parameters of the HPA axis activity were shown to be correlated to cattle temperament. Temperamental heifers were found to have higher baseline cortisol concentrations than calmer animals (Curley Jr. *et al.* 2008). A detailed investigation of the genetic correlation between behaviour and HPA axis parameters could be a valuable approach to identify relevant pathways and physiological responses resulting from the genetic predisposition of temperament.

In the discussion about genetic influences on behaviour, attention must also be paid to numerous environmental factors which are external stimuli for the expression of behaviour. As a consequence of substantial environmental effects on behaviour, genes affecting temperament in cattle are noted to have smaller effect sizes in comparison to genetic loci, which are associated with production traits, resulting in lower explanations of the phenotypic variability and estimated heritabilities (Gutiérrez-Gil *et al.* 2008). Flint (2003) found that in laboratory rodents merely 10% of behaviour differences are caused by genes. Nevertheless, genes and environment should not be considered as antagonistic factors in the regulation of behaviour, but rather as interactive (Bendesky & Bargmann 2011). However, the approach of nature and nurture in the context of behaviour is still debated as controversial in the literature.

Genomic regions associated with temperament traits

Genetic markers for behavioural characteristics have already been identified in different livestock species, for example for feather picking in hens (Flisikowski *et al.* 2009) and for different behavioural traits in pigs (Reiner *et al.* 2009). In cattle, the results of previous studies have provided further proof for a genetic disposition of behaviour and moreover confirm the assumption that specific behavioural traits are influenced by different genomic regions (Schmutz *et al.* 2001; Gutiérrez-Gil *et al.* 2008). In the following, the important analyses related to cattle temperament and genetics are summarized.

In dairy cattle, research about the genetic correlation of behaviour has been focussed on milking temperament primarily. Spelman *et al.* (1999) assessed subjective temperament scores for New Zealand Holstein-Friesian and Jersey cows during milking for genetic

analysis, but no QTL (quantitative trait loci) for milking adaptability could be identified. Likewise, Schrooten *et al.* (2000) found no QTL correlated with temperament during milking in Holstein-Friesian cattle, but three genomic regions with suggestive linkage for milking speed where located on chromosomes 2, 3 and 23. In contrast, Hiendleder *et al.* (2003) detected four QTL for behaviour during milking on the chromosomes 5, 18, and 29 in the same breed. Additionally, these QTL were in close proximity to QTL identified for milking speed in the same study, indicating that these might be single QTL affecting both traits. In further QTL mapping studies, the behaviour phenotypes were assessed during specific test conditions and other routine handling procedures. Five microsatellite markers were identified to be linked to flight distance towards unfamiliar humans in Limousin and Jersey cows by Fisher *et al.* (2001). Two more polymorphisms were associated with the cortisol concentration in urine and one putative marker was detected for plasma cortisol level as a response to stress before slaughtering. In a cross-breed population of Brahman and Angus cattle, behaviour was scored for aggressiveness, nervousness, flightiness, gregariousness and overall temperament during weaning and slaughtering. QTL for these scores were found on BTA1, 4, 8, 9, 16 and 18 (Wegenhoft 2005). Boldt (2008) analysed the same experimental population confirming the temperament associated QTL on BTA8 and found additional QTL on BTA3, 6, 12, 26 and 29 by the use of different statistical approaches. Gutiérrez-Gil *et al.* (2008) detected 29 QTL, distributed over 17 chromosomes in a Holstein-Charolais cross-breed population. These genomic regions were significantly associated with traits like frequency of vocalization, flight distance or standing at alert that were recorded during a flight from a feeder and a social separation test. In some of these behaviour related linkage studies, dominance effects of QTL were reported (Wegenhoft 2005; Gutiérrez-Gil *et al.* 2008). Aberrations concerning rearing conditions and cattle breeds (Hoppe *et al.* 2010) as well as different evaluations of behaviour phenotypes and different marker densities complicate the comparability between studies and must be taken into account. Nevertheless, overlapping QTL were found between the studies, especially on BTA29 (Hiendleder *et al.* 2003; Gutiérrez-Gil *et al.* 2008; Glenske *et al.* 2011).

Candidate genes

Another approach for revealing molecular pathways which modulate behaviour is the investigation of functional candidate genes that are associated with behavioural

characteristics underlying temperament in other species (reviewed in Bendesky & Bargmann 2011) or of positional candidate genes that are located in QTL for behavioural traits. In cattle, putative candidate genes that affect behavioural traits in distinct situations such as oestrus and feeding behaviour have been reported (Nkrumah *et al.* 2005; Kommadath *et al.* 2011; Hulsegge *et al.* 2013). One example for a positional and functional candidate gene for cattle temperament is the tyrosinase gene (*TYR*), which is generally known for its function in the dilution of coat color in cattle (Schmidtz *et al.* 2001), and is located in a QTL for temperament during milking on BTA29 (Hiendleder *et al.* 2003). Tyrosinase catalyses reactions in the dopamine metabolism and is assumed to be involved in the appearance of Parkinson`s disease in humans (Hasegawa 2010). Other genes involved in dopamine metabolism have been suggested as further functional candidate genes because the neurotransmitter dopamine itself is associated with behavioural traits and diseases in different species. A prominent candidate gene, the dopamine receptor D4 gene (*DRD4*) has been associated with behavioural traits like novelty seeking and curiosity in humans and different animals (Bailey *et al.* 2007; Munafò *et al.* 2008; Korsten *et al.* 2010). In cattle, *DRD4* can be mapped to the distal part of BTA29 (Glenske *et al.* 2011), but no QTL or direct association for temperament in cattle have been identified in this region so far. Another widely discussed functional candidate gene is the monoamine oxidase A (*MAO A*) gene, which degrades catecholamines like serotonin, norepinephrine and dopamine (Shih *et al.* 1999). Lühken *et al.* (2010) analysed the structure of the *MAO A* gene in German Angus and Simmental cattle and identified five SNPs in the coding region but none of these polymorphisms were significantly associated with behaviour scores that were assessed during tethering, weighing and social separation tests. Further positional candidate genes that were located in QTL regions associated with temperament are the cannabinoid receptor (*CNR1*) gene on BTA9 (Schmutz *et al.* 2001), the regulator of G-protein signaling 2 (*RGS2*) gene, the plexin A2 (*PLXA2*) gene on BTA16 and the prolactin precursor receptor (*PRL-R*) gene on BTA20 (Gutiérrez-Gil *et al.* 2008), but no further investigation of these candidate genes have been performed in cattle thus far.

Perspective and challenges of behaviour genetics in cattle

Increasing attention has been paid to cattle temperament in livestock production for its benefit to working safety, adaptability to new housing conditions, animal welfare and production. Boissy *et al.* (2005) even considered the importance of selection for adaptability as equal in importance to the quality of housing systems with regard to animal welfare. As a consequence, breeding for cattle behaviour has been intensively discussed. In some countries, milking temperament of dairy cattle is already integrated as a selection index into breeding programs (reviewed in Adamczyk *et al.* 2013), whereas in beef cattle, temperament is indeed recognized as an important trait for economic efficiency and frequently assessed, but its use as a selection index is uncommon (Sant'anna *et al.* 2013). Reasons for this non-consideration are the possible competitive genetic relationship between temperament and production traits (Oltenacu & Broom 2010) and complex behaviour evaluations.

To date considerable insights into behaviour genetics from candidate genes to key neurological pathways have been given for other species (reviewed in Bendesky & Bargmann 2011), but information on cattle are limited to QTL mapped for behaviour, which still need confirmation and functional approval. To overcome this lack of information, further research is needed taking new technologies, such as microarrays, next generation sequencing and metabolomics, into account. In addition, objective and informative methods for the assessment of cattle temperament are needed, which can then be standardized for use in cattle husbandry and breeding. In general, the behaviour measurement should have adequate heritability, a high level of reproducibility, simple application and should include handling conditions since approachability or fear of humans are important aspects of cattle behaviour.

Chapter 3 Detection of genetic variants affecting cattle behaviour and their impact on milk production – a genome-wide association study

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Detection of genetic variants affecting cattle behaviour and their impact on milk production – a genome-wide association study

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Abstract

Behaviour traits of cattle have been reported to affect important production traits, such as meat quality and milk performance as well as reproduction and health. Genetic predisposition is, together with environmental stimuli, undoubtedly involved in the development of behaviour phenotypes. Underlying molecular mechanisms affecting behaviour in general and behaviour and productions traits in particular still have to be studied in detail. Therefore, we performed a genome-wide association study in an F_2 Charolais x German Holstein cross-breed population to identify genetic variants that affect behaviour-related traits assessed in an open-field and novel-object test and analysed their putative impact on milk performance. Of 37,201 tested single nucleotide polymorphism (SNPs), four SNPs showed a genome-wide and 37 a chromosome-wide significant association with behaviour traits assessed in both tests. Nine of the SNPs that were associated with behaviour traits likewise showed a nominal significant association with milk performance traits. On chromosomes 14 and 29, six SNPs were identified to be associated with exploratory behaviour and inactivity during the novel-object test as well as with milk yield traits. Least squares means for behaviour and milk performance traits for these SNPs revealed that genotypes associated with higher inactivity and less exploratory behaviour promote higher milk yields. Whether these results are due to molecular mechanisms simultaneously affecting behaviour and milk performance or due

to a behaviour predisposition, which causes indirect effects on milk performance by influencing individual reactivity, needs further investigation.

Keywords: GWAS, behaviour genetics, milk performance, novel-object test, open-field test

Introduction

In farm animal research, there is growing interest in the identification of genetic variations and molecular mechanisms which affect behaviour, as behaviour has been shown to have an impact on economically important production traits. In beef cattle, for example, calmness or adaptability is favourably correlated with daily weight gains and meat quality (Hall *et al.* 2011; Vetters *et al.* 2013). Other authors showed a negative effect of insufficient coping adaptabilities on novel situations, such as milking in an unfamiliar milking parlour, on milk yield in dairy cattle (Rushen *et al.* 2001; Sutherland *et al.* 2012).

Behaviour is known to be modulated by environmental factors as well as by genetic predisposition. A genetic background of cattle behaviour is indicated by breed differences (Gauly *et al.* 2002) and estimated moderate to high heritabilities for temperament traits (Gauly *et al.* 2001; Benhajali *et al.* 2010; Sewalem *et al.* 2011; Riley *et al.* 2014). In addition, several studies have mapped quantitative trait loci (QTL) for behaviour-related traits, such as habituation to new situations and flight distances, using microsatellite markers (Schmutz *et al.* 2001; Hiendleder *et al.* 2003; Gutiérrez-Gil *et al.* 2008). Recent studies using single nucleotide polymorphism (SNPs) identified behaviour-associated genetic variance (Kramer *et al.* 2014; Hulsman Hanna *et al.* 2014). Information about the impact of behaviour on production traits derives mainly from studies investigating their phenotypic correlation. Only a few studies have mapped QTL for temperament and milking speed (Kolbehdari *et al.* 2008) and showed an overlap in QTL positions (Hiendleder *et al.* 2003). A mutual link between behaviour and the actual trait milk performance has not been analysed yet.

To further investigate the genetic background of cattle behaviour, we performed a genome-wide association study for behaviour phenotypes measured in open field (OF) and novel-object (NO) tests. Additionally, the putative impact of SNPs associated with

behaviour on milk yield and the effect of rehousing on milk performance were analysed. Animals used for analysis derived from a segregating F_2 population of a cross between German Holstein and Charolais which showed deviations from typical lactation curves (Hammon *et al.* 2007) and high variance in behaviour (Graunke *et al.* 2013).

Materials and methods

Animals

A total of 147 F_2 cows derived from a cross between German Holstein and Charolais (SEGFAM, Kühn *et al.* 2002) were analysed. All animals were reared at the Leibniz Institute for Farm Animal Biology in Dummerstorf, Germany, under standardised feeding and husbandry conditions (Hammon *et al.* 2007). Calves were weaned immediately after birth and housed in group pens. To avoid animal-specific behaviour differences resulting from individual experiences, rearing and behaviour testing were standardised. The experimental procedures were carried out according to the animal care guidelines of the State Mecklenburg-Vorpommern, Germany, and were approved by the relevant authorities.

Behaviour traits

An OF and a NO test were performed for each calf at the age of 90 ± 3 days *post natum*. The testing procedure is described in detail in Graunke *et al.* (2013). First, the calves could habituate to an open field arena (9.6 m x 4.0 m) for 10 min, which is referred to here as OF, subsequently, for the NO test, a traffic cone was placed in the arena for another 10 min. During the test, several behaviour parameters were recorded, and the duration of calves being active (DA; in s), inactive (DI; in s), and exploratory towards the arena (DE; in s) was selected as phenotypes for analyses.

Milk traits

Immediately after calving, the cows were milked in a tie stall barn for approximately 5 days and subsequently rehoused to a loose stall barn with a conventional tandem milking parlour. In both barns, cows were milked twice a day. Lactation characteristics of the F_2 SEGFAM cows were previously described in Hammon *et al.* (2007). Considerable differences could be observed regarding length and milk yield in the first

lactation, especially between day 30 and day 100. The milk yield (in kg) during the first 5 days (MY5) and from day 6 to day 30 (MY30) as well as the average milk yield (MY; in kg/day) from day 1 to the end of the lactation was considered as milk performance traits. To quantify the response to rehousing in regard to milk yield, the ratios between the milk yield 1 day before and 1 day after rehousing (R1) and 3 days before and after rehousing (R3) were calculated. R1 and R3 were selected for analyses because the drop in milk yield observed after rehousing could be related to the individual reactivity to novelty as described by Sutherland *et al*. (2012). Descriptive statistics for the analysed traits are provided in Table 3.1.

Table 3.1 Descriptive statistics of analysed behavioural and milk performance traits

Trait	Test	n	Mean	SD
DI[a]	OF	147	322.5	72.0
	NO	147	429.0	49.5
DA[a]	OF	147	122.8	40.1
	NO	147	84.0	49.5
DE[a]	OF	147	125.9	49.6
	NO	147	65.8	50.3
MY[b]		147	5.33	4.31
Y5[b]		147	26.7	12.75
Y30[b]		139	175.6	125.1
R1[c]		144	1.18	0.56
R3[c]		144	1.28	0.74

[a]in seconds (maximum 600s)

[b]in kg

[c]ratio

DNA extraction and SNP Genotyping

DNA was extracted from mammary gland tissue with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. The samples were genotyped using the Illumina BovineSNP50 BeadChip v1.0 and v2.0 (Illumina Inc., San Diego, CA, USA). Genotype data pre-processing and quality control (Infinium Genotyping Data Analysis; http://www.illumina.com/Documents/products/technotes/technote_infinium_genotyping_data_analysis.pdf) were realised via GenomeStudio® Software V2011.1 (Illumina Inc., San Diego, CA, USA). Quality metrics were checked twice for each array version

separately, and conspicuous SNPs were reclustered or excluded. The samples were filtered for a minimum call rate of 95%. After merging the v1.0 and v2.0 data sets, SNPs with a minor allele frequency of < 5% and more than 10% missing genotypes were removed from the data sets. To identify inconsistencies between recorded genotypes and pedigree information, the software PedCheck (O'Connell & Weeks 1998) was used. The final dataset comprised 37,201 SNPs.

Statistical analyses

Correlation between behaviour and milk traits: To study the phenotypic relationship between behaviour and milk performance traits, pairwise Spearman rank correlation was calculated in JMP Genomics software version 5.1 (SAS Institute Inc., USA).

GWAS for behaviour traits: A GWAS was conducted for each of the behaviour traits separately. The additive SNP effect on the three behaviour traits was modelled in Qxpak 5.05 (Pérez-Enciso & Misztal 2011):

$$y_{ijk} = \mu + YS_j + a_k + u_i + e_{ijk}$$

where y_{ijk} is the behaviour trait of animal i ($i = 1, \dots, 147$), μ is the overall mean, YS_j is the fixed effect of year and season ($i = 1, \dots, 22$), a_k is the fixed additive-genetic effect of each SNP k ($k \in \{1, \dots, 37{,}201\}$), u_i is the random infinitesimal polygenic effect of animal i estimated from pedigree information and the residual random effect is $e_{ijk} \sim N(0, \sigma_e^2)$. To avoid the detection of false positives due to multiple testing, a conservative 5% genome-wide and chromosome-wide significance threshold was applied using Bonferroni correction.

Association of SNPs with milk performance and behaviour: A QK mixed model, which adjusts for family relatedness (Yu *et al.* 2006) was applied to test for associations between milk performance traits and SNPs we identified in the GWAS to be significantly associated with behaviour traits. Year and season at onset of the first lactation or the day of behaviour tests were considered as fixed effect for milk performance and behaviour traits respectively. Additionally, least squares means by

genotype were calculated for SNPs that were simultaneously significantly associated with milk performance and behaviour traits.

In addition, specific linkage disequilibrium (LD) blocks were investigated across chromosomal positions of interest, using LD block creation (JMP Genomics 5.1). Therefore, *D'* and the r² coefficient were used to estimate LD between a pair of SNPs. The LD measure *D'* was used subsequently to create blocks of SNPs that were in strong LD (Gabriel *et al.* 2002).

Results

Correlation between behaviour and milk performance traits

The highest correlations were observed between traits of the particular phenotype group (behaviour or milk performance). Behaviour and milk performance traits showed no significant correlations, except for DE (NO test) and milk yield traits (MY, Y5, Y30), which were negatively correlated (r_s = -0.18, -0.20 and -0.17; *P*-value < 0.05). The ratios R1 and R3, which indicate the responsiveness towards rehousing visible in milk yield, were negatively correlated to MY (r_s = -0.38 and -0.60), Y5 (r_s = -0.31 and -0.35) and Y30 (r_s = -0.42 and -0.63) with *P*-value < 0.001.

GWAS for behaviour traits

Of the 37,201 SNPs, four on chromosomes 2, 10 and 19 were associated with the behaviour traits DI (OF and NO test) and DE (NO test) at the 5% genome-wide significance level ($P\text{-value}_{genome} = 1.34 \times 10^{-6}$). In addition, significant associations were detected for all analysed behaviour traits at the chromosome-wide significance level ($P\text{-value}_{chromosome} = 1.08 \times 10^{-4}$ to $P\text{-value}_{chromosome} = 2.15 \times 10^{-5}$). *P*-values, chromosomal position and estimated SNP effects for the four genome-wide and 37 chromosome-wide significant SNPs are provided in Table 3.2. The 41 significant markers were distributed over 21 chromosomes. Twenty-six SNPs were identified to affect behaviours in the NO test, whereas 15 SNPs were associated with behaviour assessed during the OF test. On BTA10 and 14, SNPs were simultaneously significant for the negatively correlated traits DI and DE (NO test). Inverse SNP effects of these SNPs (Table 3.2) are in agreement with the negative phenotypic correlation observed between these traits, similar for the significant SNP associated with DA and DI on BTA26 (NO test). According to SNP

positions, 19 of the significant SNPs could be assigned to genes (*Bos taurus* UMD3.1; NCBI map viewer; Annotation Release 103; http://www.ncbi.nlm.nih.gov/mapview/).

Table 3. 2 Significant SNPs associated with behavioural traits

SNP name	**Chr**	**Position (bp)**	**Trait**	***P*-value**	**Gene**	**SNP effect**	**SE**
			open-field test				
rs109193448	2	62447051	DI	$6.09x10^{-7}$**	-	-47.28	9.14
rs111021714	2	62890977	DI	$5.65x10^{-7}$**	*TMEM163*	-51.34	9.94
rs43332694	2	69774359	DI	$1.35x10^{-5}$*	-	-71.39	16.12
rs41255467	6	69184582	DE	$2.05x10^{-6}$*	*OCIAD2*	-31.59	6.13
rs109064778	8	98532984	DA	$2.95x10^{-6}$*	-	-29.90	6.18
rs108979436	14	8984849	DI	$2.21x10^{-6}$*	*ST3GAL1*	44.41	9.17
rs29019596	19	38157880	DI	$2.94x10^{-5}$*	*IGF2BP1*	-67.91	16.01
BTA-12468-no-rs	21	9375095	DA	$8.61x10^{-6}$*	*LOC782362*	-26.79	7.46
rs110780905	21	9888915	DA	$3.73x10^{-5}$*	-	31.58	5.83
rs109674592	22	52113096	DI	$3.70x10^{-5}$*	*SPINK8*	-35.55	9.68
rs110027993	22	52827405	DI	$2.81x10^{-5}$*	*PTPN23*	-41.25	8.57
rs29012505	24	920520	DI	$1.93x10^{-5}$*	-	44.39	10.18
Hapmap47669-BTA-59022	24	1094942	DI	$1.55x10^{-5}$*	*NFATC1*	42.13	9.62
rs109679723	24	21649461	DA	$8.82x10^{-5}$*	-	-31.36	6.84
			novel-object test				
BTA-122016-no-rs	3	38811314	DE	$1.24x10^{-5}$*	-	-26.61	5.70
rs29027498	4	26490406	DE	$1.28x10^{-5}$*	-	-29.35	6.52
rs43708473	7	112133700	DA	$1.24x10^{-5}$*	*TMEM232*	35.34	7.93
rs109313646	9	85002515	DE	$1.81x10^{-5}$*	*LOC781799*	27.90	6.20
rs111019360	9	94683820	DE	$2.26x10^{-5}$*	-	27.81	6.25
rs110025880	10	20008636	DA	$6.41x10^{-6}$*	*HCN4*	-56.55	12.18
rs41256789	10	46312239	DE	$1.96x10^{-6}$*	-	-62.16	12.6
rs109741931	10	46548679	DE	$1.37x10^{-6}$*	*USP3*	-66.28	13.22
rs42838073	10	46665928	DI	$2.86x10^{-5}$*	-	76.95	17.73
			DE	$9.35x10^{-6}$*	-	-44.28	9.77
Hapmap31150-BTA-152385	10	66687859	DE	$9.99x10^{-7}$**	-	41.45	8.15
Hapmap48681-BTA-19661	12	33721124	DI	$1.08x10^{-5}$*	*ATP8A2*	46.36	10.22
rs109784719	14	44941560	DI	$3.28x10^{-6}$*	-	-47.6	9.88
			DE	$1.48x10^{-6}$*	-	24.64	5.52
rs110245129	14	68599010	DI	$1.56x10^{-5}$*	*MATN2*	-47.57	10.70
rs41666787	14	71025573	DI	$2.72x10^{-5}$*	-	58.25	13.52
rs109494085	15	53250782	DE	$2.67x10^{-5}$*	*FCHSD2*	-54.52	12.32
rs109513733	16	50798300	DE	$1.88x10^{-5}$*	*MEGF6*	-29.61	6.72

rs17597495	19	17597495	DE	$1.08x10^{-5}$*	-	35.78	7.71
Hapmap38959-BTA-44727	19	21950487	DE	$3.21x10^{-5}$*	-	-31.93	7.28
rs110894302	19	22157176	DI	$2.52x10^{-7}$**	-	74.43	13.78
rs109243151	25	10305794	DA	$3.00x10^{-5}$*	*LITAF*	-44.32	10.30
rs110898125	26	48583446	DI	$7.97x10^{-6}$*	-	-61.62	13.21
			DA	$3.44x10^{-5}$*	-	31.32	7.35
rs42138859	28	24751304	DA	$2.94x10^{-5}$*	*MYPN*	-30.57	7.20
rs108965864	29	19234709	DE	$4.27x10^{-5}$*	*LOC524642*	-33.50	7.77
rs42169108	29	19332326	DE	$4.27x10^{-5}$*	-	-33.50	7.77
rs43099931	29	19376416	DE	$4.27x10^{-5}$*	-	-33.50	7.77
rs29025765	X	15953283	DA	$8.18x10^{-5}$*	-	-35.62	8.93

Significant for a chromosome-wide (*) and genome-wide (**) Bonferroni-corrected *P*-value of = 0.05

Association of SNPs with milk performance and behaviour

In total, nine of the 41 SNPs associated with behaviour traits were also associated with MY, Y5, Y30, R1 or R3 at a nominal significance level of *P*-value < 0.05 (Table 3.3). Only SNPs affecting behaviour traits assessed in the NO test were observed to putatively affect milk performance traits. These SNPs were located on BTA7, 10, 14, 19 and 29 and six of them were associated with more than one milk performance trait. For SNPs significantly associated with behaviour and milk performance traits, our results indicate competitive genotype effects in regard to active and exploratory behaviour with milk yield. Genotypes associated with higher inactivity were associated with higher milk yield and less response to rehousing. This relationship is prominently reflected by genotype effects for the three SNPs on BTA29 (rs108965864, rs42169108, rs43099931) at the position of approximately 19.3 Mbp which were in full LD in a region of 418,005 bp ($r^2 = 1$). Animals carrying the genotype combination "AA-AT-AA" had on average higher milk yield (MY +2.53 kg/day; Y30 +66.04 kg), whereas the milk yield response towards rehousing was lower (R3 -0.41). Additionally, in comparison to cows with the "AG-TT-AG" combination, they spent less time exploring the arena (DE NO test -36s).

Table 3.3 Least squares means ± standard error (LSM ± SE) by genotype for single nucleotide polymorphism (SNPs) which are simultaneously significantly associated with behaviour traits in the novel-object test (NO) and milk performance traits

SNP	**Chr**	**Trait**	**Genotype**					
			0		**1**		**2**	
			n	**LSM±SE**	**n**	**LSM±SE**	**n**	**LSM±SE**
rs43708473	7	Y5	72	26.1±1.5	72	30.9±1.6*	3	2.0±7.3**
		Y30		189.2±15.3		194.7±15.9		-16.4±70.9*
		DA		103.1±5.5		66.2±5.5**		54.4±26.8
rs110025880	10	Y5	130	28.2±1.2	16	25.0±3.3	1	12.8±4.3*
		DA		75.5±15.3		128.7±19.0**		194.4±48.0*
rs109784719	14	MY	56	4.3±0.6	58	6.1±0.6*	33	7.4±0.8**
		R1		1.4±0.1		1.1±0.1		1.0±0.1**
		DI		393.1±22.1		441.2±22.0*		479.5±25.1**
		DE		86.3±8.4		57.8±8.4**		37.5±10.4**
rs110245129	14	R1	41	1.4±0.1	73	1.2±0.1*	33	0.9±0.2**
		R3		1.6±0.2		1.4±0.2		0.9±0.2**
		DI		394.1±13.4		418.3±10.0		496.3±14.9**
rs41666787	14	Y5	76	31.0±1.7	65	25.1±1.6*	6	26.5±5.1
		DI		442.0±20.7		414.5±21.5		355.1±42.4*
Hapmap38959-BTA-44727	19	Y5	27	33.2±3.0	101	27.8±1.9	19	22.5±3.3*
		DE		33.1±16.0		67.0±13.3**		91.7±16.6**
rs108965864	29	Y30	90	211.8±13.1	57	145.8±17.5**		
		MY		6.5±0.8		3.9±0.8**		
		R3		1.2±0.1		1.6±0.2**		
		DE		51.0±14.2		87.0±14.6**		
rs42169108	29	Y30	90	211.8±13.1	57	145.8±17.5**		
		R3		1.2±0.1		1.6±0.2**		
		MY		6.5±0.8		3.9±0.8**		
		DE		51.0±14.2		87.0±14.6**		
rs43099931	29	R3	90	1.2±0.1	57	1.6±0.2**		
		MY		6.5±0.8		3.9±0.8**		
		Y30		211.8±13.1		145.8±17.5**		
		DE		51.0±14.2		87.0±14.6**		

0, Homozygote major allele

1, Heterozygote

2, Homozygote minor allele

Significantly different from genotype 0 for *P*-value = 0.05 (*); *P*-value = 0.005 (**)

Discussion

In the present study, we analysed the association of genetic variants with cattle behaviour traits by performing a GWAS using SNP markers. The behaviour traits DA, DE and DI were selected for analyses due to their significance in the particular testing situation as described in Réale *et al*. (2007) and because they were shown to be the most informative traits in the assessment of temperament types in the study of Graunke *et al.* (2013). In addition, the milk traits MY, MY5 and MY30 and the response to rehousing regarding milk yield (R1 and R3) were analysed to evaluate whether SNPs affecting behaviour have an impact on milk performance. Despite the small population size that affected the power of this study, we were able to identify several genomic regions that were associated with behaviour traits. SNPs on BTA2, 9, 10, 14, 19 and 29 that affected the same or negatively correlated traits within and across test situations provided strong evidence for a genetic background of cattle behaviour. Despite the correction for population stratification by including a pedigree-based relationship matrix in the models, Lambda values, ranging from 1.28 for DA (NO test) to 1.45 for DE (NO test), indicated an intermediate genome-wide inflation of *P*-values. Genomic inflation was shown to be likely under polygenic inheritance and could be further affected by the small sample size and a substantial LD observed in this study, for example, on BTA29 (Yang *et al.* 2011; Höglund *et al.* 2014).

All behaviour traits analysed were affected by more than one SNP, and SNPs affecting behaviour traits were found on 21 chromosomes, confirming that behaviour is a quantitative trait affected by numerous genetic loci (Jensen 2006). Most of the SNPs affecting behaviour traits in the different test situations were located on different chromosomes. This could be attributed to different strategies used to cope with the different challenges in the two test situations.

Although a comparison to other studies investigating the genetic background of cattle behaviour is hindered by the use of different behaviour traits and breeds as well as by different rearing conditions and marker mapping strategies, we were able to identify SNPs affecting behaviour traits in genomic regions that were previously reported to affect behaviour- or temperament-related traits in other studies. SNPs on BTA4, 6, 7, 8, 9, 10, 14, 15, 16, 19, 21, 25, 26, 28 and 29 were located in regions that were previously

identified to affect behaviour- or temperament-related traits (Schmutz *et al.* 2001; Hiendleder *et al.* 2003; Gutiérrez-Gil *et al.* 2008; Glenske *et al.* 2011), whereas SNPs on chromosomes 2, 3, 12, 21, 22, 24 and X were located in genomic regions that have not been reported previously. Gutiérrez-Gil *et al.* (2008) investigated a Charolais x Holstein cross-breed population using microsatellite markers, and phenotypes recorded during a flight from feeder and a social separation test. They identified a total of 29 QTL, and seven of these were located in regions which are coincident to our study. In the study of Gutiérrez-Gil *et al.* (2008), most of the QTL were related to vocalisation events which were not investigated in our study. However, the behaviour traits of walking, escaping and running (WER) or standing alert (SA) represent traits that are closely related to the activity measurements analysed in our study. QTL regions for WER and SA overlapped with the position of SNP associated with DA (OF test) on BTA8, DE (NO test) on BTA9 and DI (OF test) on BTA19.

Other studies used temperament assessed in different situations as behavioural phenotypes. Schmutz *et al*. (2001), for example, identified a QTL for temperament during handling on BTA14 in beef cattle that is located in the same region where we identified a SNP to be associated with DI and DE. Hulsman Hanna *et al.* (2014) detected the same SNP on BTA 14 (rs41666787) that was associated with DI in our study to be putatively associated with temperament during weaning in a Nellore x Angus cross-breed population. They also found strong evidence for a QTL for temperament on BTA29 located upstream of the three SNPs identified in our study. For BTA29, several studies found indication for a genetic effect on behaviour or temperament related traits (Hiendleder *et al.* 2003; Gutiérrez-Gil *et al.* 2008; Kolbehdari *et al.* 2009; Glenske *et al.* 2011; Hulsman Hanna *et al.* 2014). Hiendleder *et al.* (2003), for example, identified a QTL for temperament assessed during milking in Holstein cows in the same region where the three SNPs we identified to be associated with DE and the milk traits MY, Y30 and R3 were located and where Viitala *et al.* (2003) detected a QTL for milk yield in Finnish Ayrshire dairy cattle. Within this particular region, three genes were localised: *LOC524642* (glycerophosphodiester phosphodiesterase domain containing 4-like), *LOC100849541* (glycerophosphodiester phosphodiesterase domain containing protein 4-like, pseudogene) and *LOC782090* (eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa pseudogene). Proteins especially from the transmembrane protein family glycerophosphodiester

phosphodiesterase are known to catalyse reactions in the glycerophospholipid metabolism, which has been shown to be important for the fatty acid metabolism related to milk synthesis in lactating cows in a transcriptome profiling study (Bionaz *et al.* 2012). In cattle, few putative positional candidate genes underlying behaviour modulations, such as *DRD4* (Glenske *et al.* 2011) and *TYR* genes as well on BTA29, have been reported yet, but the positions of these genes do not overlap with SNPs identified in this study (Hiendleder *et al.* 2003). Other putative positional and functional candidate genes in the present study are genes that have been reported to be involved in neurological developmental processes or mood disorders in humans, such as *TMEM163* on BTA2 (Hoerder-Suabedissen *et al.* 2009), *HCN4* on BTA10 (Kelmendi *et al.* 2011) and *ST3GAL1* on BTA14 (Kim *et al.* 2013).

Genotype effects of SNPs significantly associated with behaviour and milk performance imply a competitive relationship of active and exploratory behaviour with milk yield. Other studies could show a suppressive effect of fear or stress on milk yield, especially in response to novelty (Sutherland *et al.* 2012). We assume that cows expressing high levels of active and exploratory behaviour are in an agitated condition, which is supported by heart rate variability measurements of our experimental animals (Graunke *et al.* 2013). Agitated behaviour could restrain milk production due to stress supressing oxytocin release or the hormone effects, resulting in a disruption of milk removal (Bruckmaier & Blum 1998).

In conclusion, we were able to identify several genetic loci affecting different behaviour traits during two test situations. Inverse effects of genotypes of significant SNPs between agitation (activity, exploration) and milk performance traits indicate that selection for high milk yields likewise promoted the selection of animals with a lower reactivity towards novelty. It remains unclear whether there are genetic loci affecting both milk performance and behaviour or if the behaviour predisposition itself is responsible for the differences in regard to milk yield as indicated by the differences in the response to rehousing.

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Chapter 4 Differences in gene expression profiles of cattle with distinct temperament types

Submitted

Differences in gene expression profiles of cattle with distinct temperament types

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Abstract

Temperament affects ease of handling, animal welfare, and economically important production traits in cattle. Several recent studies have reported a link between cattle behaviour, physiological parameters (for example cortisol concentration or heart rate variability) and production traits. However, knowledge about the complex biological architecture of cattle temperament is still limited. In this study, differences in gene expression profiles of the adrenal cortex between 60 F_2 cows (Charolais x German Holstein) of distinct temperament types were analysed at the time of slaughter in the second lactation at an age of 1309±105 days. The cows were assessed in a novel-human test at an age of 90 days. Genes that were significantly differentially expressed between temperament types were identified by ANOVA. The differences in the expression profiles seemed to be mainly triggered by fear because the greatest differences were observed between the "fearful/neophobic-alert" and all other temperament types. The significantly differentially expressed genes highlight the importance of adrenal cortex development and individual stress reactivity as well as immune function regarding cattle temperament. Thus, genes involved in functional processes, such as cellular maintenance, proliferation and survival, or pathways related to the stress response (for example 'NRF2-mediated Oxidative Stress' and immune related pathways) were

differentially expressed among temperament types. Overall, the present study provides new insight into transcriptional differences in the adrenal cortex between cows of distinct temperament types, further supporting the assumption of a relatively stable molecular response to stressful situations in livestock species.
Keywords: temperament, cattle, adrenal cortex, stress

Introduction

Cattle temperament, which is a multidimensional and complex biological trait (Réale *et al.* 2007), is characterized by stable behaviour and physiological responses to challenging situations (Sutherland *et al.* 2012). The response to challenging conditions could differ between individuals (Koolhaas *et al.* 2010) and be manifested in behaviour characteristics, such as shyness-boldness, exploration-avoidance, activity, sociability, and aggressiveness (Réale *et al.* 2007). Cattle temperament is of growing interest for society and the cattle industry because of its impact on handling safety, animal welfare and economically important production traits (Hemsworth *et al.* 2000; Nkrumah *et al.* 2007; Hall *et al.* 2011; Vetters *et al.* 2013).

Environmental conditions and individual experiences are known to affect behavioural characteristics, but environment-gene interactions and endogenous processes contribute to the behavioural phenotype as well. The underlying physiological mechanisms of behaviour are mediated by complex response networks characterized by neurophysiological and molecular factors including hormones and proteins, which are also affected by gene expression (Johnston & Edwards 2002). In cattle, the HPA axis and sympathomedullary pathway (SAM) are well recognised in the physiological response to challenging situations and for their association with behavioural traits and temperament in cattle (King *et al.* 2006; Curley Jr. *et al.* 2008; Cafe *et al.* 2011a; Burdick *et al.* 2011b). Parameters related to glucocorticoid and catecholamine biosynthesis, for example, cortisol and epinephrine concentration, were observed to be affected by stress during transportation and allowed for the differentiation of calm and temperamental cattle (Burdick *et al.* 2011a). In beef cattle, a higher responsiveness towards handling is associated with a higher basal concentration of glucocorticoids

(Curley Jr. *et al.* 2008), suggesting a general higher activation of the HPA axis in more excitable cattle. Furthermore, it has been shown that stress during weaning can significantly alter transcriptome profiles in the leucocytes of calves, especially the expression of transcripts involved in G-protein coupled receptor (GPCR) signalling (O'Loughlin *et al.* 2012). Such findings demonstrate the importance of gene expression and molecular networks in the manifestation of behavioural characteristics.

The analysis of comprehensive '-omics' profiles allows researchers to study the molecular mechanisms underlying complex biological processes (Joyce & Palsson 2006). In the context of cattle temperament, a study of the transcriptome could enable the identification of relevant pathways and regulatory networks related to temperament in different tissues. Although circuits of the limbic system, for example, the amygdala and hippocampus, are frequently investigated for their function in the emotions and cognitive ability of various species, insights into other tissues are rare. The adrenal gland has a pivotal role in the endocrinology of stress via the synthesis of stress-related hormones, i.e., cortisol, epinephrine and norepinephrine (Charmandari *et al.* 2005). Thus, the adrenal cortex has a function in behaviour development in mammals as a part of the HPA axis (glucocorticoid synthesis) (reviewed in Brain 1972). Previous work on adrenal gene expression in chickens showed that differences in the ACTH sensitivity of the adrenal glands might play a pivotal role in the variability of the stress response (Bureau *et al.* 2009).

Therefore in this study, we analysed the bovine adrenocortical transcriptome at slaughter, when animals were subjected to different environmental stressors such as novelty, social separation and human handling (Terlouw *et al.* 2012), for differences in gene expression profiles between temperament types assessed in a novel-human test to identify molecular mechanisms as potential targets for further research into cattle temperament. The data presented here show that temperament types assessed in response to environmental challenges differ in adrenocortical gene expression at slaughter. The transcriptional differentiation of temperament types is primarily related to genes involved in adrenal cellular processes, stress response pathways and immune function.

Materials and methods

Animal husbandry

We analysed 60 F_2 cows of a cross between Charolais x German Holstein cattle (SEGFAM; Kühn *et al.* 2002). All animals were reared and housed in a loose housing barn at the Leibniz Institute for Farm Animal Biology (FBN) in Dummerstorf, Germany, under the same environmental and feeding conditions (Hammon *et al.* 2007). To avoid animal-specific behavioural differences resulting from individual experience, rearing and behavioural testing were standardised. The experimental procedures were carried out according to the animal care guidelines of the State Mecklenburg-Vorpommern, Germany and were approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (Reference number: LVL M-V/310-4/7221.3-2.1-017/03).

Behaviour test and temperament types

At the age of 90 days, all calves were subjected to a novel object and a novel human test in a 9.6 m x 4.0 m open field that was divided in four segments of 2.4-m length. The age of 90 days was selected for behavioural testing because calves could develop individual behavioural characteristics based on experiences and to ensure comparability because the calves were regrouped afterwards. A detailed description of the experimental setup, the behaviours recorded and the analyses performed to assess the temperament types is given in Graunke *et al.* (2013) for the novel object test. The novel human test was performed in accordance with the novel object test after the novel object test by exchanging the novel object (a traffic pylon) with a staff person unknown to the calf. Briefly, measurements of behaviours were live-recorded during the two tests, which lasted ten minutes each, using the observation software tool The Observer 5.0 (Noldus, The Netherlands). The behaviours recorded were contact with the novel object or human, inactivity, exploration, grooming, activity, running, vocalization, changes between segments, the habituation of the calf in the open field segment harbouring the novel object or human and the habituation of the calf in the neighbouring segment.

For this study, only the temperament types assessed for the novel human test were used. Therefore, the data were analysed using a principal component analysis (PCA) (Brand *et al.* 2015). The first two principal components (PC) explained 45.0% and 16.9% of the

variance in the novel human test and were predominantly influenced by behaviours comprising contact with the novel human and the time spent near the human (PC1) and by the exploration of the open field and the inactivity of the calves during the test (PC2). The loadings of PC1 and PC2 are shown in Table S4.1. Based on the PC-scores of PC1 and PC2 that were calculated for each calf, the calves were assigned into nine groups. In regard to the exploration and avoidance of the novel human (PC1), the inactivity and exploration of the open field (PC2) and the heart rate variability, four extreme phenotypes were identified, which were described as "fearful/neophobic-alert" (low PC1-scores and high PC2-scores, TT1, n = 12), "interested-stressed" (high PC1- and PC2-scores, TT2, n = 8), "outgoing/neophilic-alert" (high PC1-scores and low PC2-scores, TT3, n = 17) and "subdued/uninterested-calm" (low PC1- and PC2-scores, TT4, n = 8) (Graunke *et al.* 2013). The animals in the fifth group showed no distinct response and were described as indistinct (TT5, n = 15). The remaining four groups were intermediate with the four extreme phenotypes and were not considered in further analyses.

Tissue sampling and RNA extraction

The cows were slaughtered at 30 days postpartum within the second lactation at an age of 1309 ± 105 days. The slaughtering procedure was standardised for all animals. In addition to other tissues, the adrenal glands were immediately taken after slaughter and were further dissected to separate the adrenal cortex from the medulla. Adrenocortical tissue samples were cut into small pieces, immediately frozen and stored at -80°C or in liquid nitrogen. Total RNA was isolated from adrenal cortex tissue using 1mL TRI Reagent (Sigma, Taufkirchen, Germany). Subsequently, the RNA was further purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The RNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany), and the integrity of the RNA was checked by running 1µg of RNA on a 1% agarose gel. To exclude DNA contamination, PCR of the glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) gene was performed using the RNA as template.

Adrenocortical gene expression profiling

For adrenocortical expression profiling, the custom Affymetrix® GeneChip® Bovine Gene v1 Array (Affymetrix, Santa Clara, USA) was used. The design of the GeneChip®

Bovine Gene v1 Array was based on Ensemble and RefSeq predictions for the Genome Bos Taurus Built 4.0. The design was targeted to develop a whole genome expression array with approximately 25 probes per transcript distributed over the entire length of each transcript. In total, the array contains 194,712 probe sets that represent almost 24,000 bovine transcripts. In addition, standard Affymetrix controls for hybridization, labelling efficiency and non-specific binding were included on the array.

For hybridization, 500ng of total RNA were amplified using an Ambion® WT Expression Kit (Ambion, Grand Island, NY, USA). Samples containing 5.5µg of the resultant cDNA were fragmented and labelled using the Affymetrix® GeneChip® WT Terminal Labelling Kit and subsequently hybridised to the microarray using the Affymetrix® GeneChip® WT Hybridization Wash and Stain Kit following the Affymetrix standard protocols. The fluidic station protocol FS540_0001 was used. Scanning was performed using the GeneChip® Scanner 3000 7G system (Affymetrix, Santa Clara, USA). Quality control was performed using the Expression Console 1.2 software (Affymetrix, Santa Clara, USA) in accordance with an Affymetrix technical note (Affymetrix 2007). The robust multi-array average (RMA) algorithm was applied for background adjustment, quantile normalisation, and summarisation. For the identification of the expressed transcripts, the detection above background (DABG) algorithm was used (*P*-value < 0.01). Probe sets were filtered for their presence in at least 75% of the animals and transcripts in which ≥ 50% of the probe sets were present were considered for further analyses. Out of the approximately 24,000 bovine transcripts represented by this array, a total of 10,986 transcripts remained after filtering and quality control. The annotation used in this study was based on the UMD3.1 assembly and all results are reported for the annotated transcripts only.

Data analyses

Because the purpose of the study was to show differences between gene expression profiles in cows assigned to distinct temperament types, an ANOVA was conducted using the following model:

$$y_{ijkl} = \mu + \beta age_i + YS_j + S_k + TT_l + e_{ijkl}$$

We considered the interaction of year and season ($YS; j = 1, \dots, 16$), sire ($S; k = 1, \dots, 3$), the linear regression of age with β as the corresponding regression coefficient (βage) of each animal i and the particular temperament type ($TT; l = 1, \dots, 5$) as fixed effects. To test for specific transcripts affected by behaviour, the estimated group means of gene expression for each temperament type were compared using Tukey`s test. To account for multiple testing, P-values were adjusted according to FDR. Mixed model analyses were performed with JMP Genomics 5.0 software (SAS Institute, NC, USA). Transcripts were filtered for FDR-adjusted P-values ≤ 0.05. Two-dimensional hierarchical clustering of gene expression was performed using the gplots package in R Version 3.1.0 (R Core Team 2014) to visualize the differences in expression of relevant genes between the temperament types.

Pathway analysis

The Ingenuity® Systems Pathway Analysis (IPA; Ingenuity Systems, Redwood City, USA; http://www.ingenuity.com) was used to perform functional analyses with the Affymetrix® Bovine Gene® 1.0 ST Array genes as a reference set, due to its high similarity to the custom array. The likelihood for the association between a set of transcripts and the assigned biological function, network, or pathway was estimated by applying a right-tailed Fisher's exact test (*P*-value = 0.05). The nominal *P*-value was calculated by considering the number of present focus genes and total genes related to known transcripts of the reference set linked with that process.

Results

Identification of transcripts differentially expressed between temperament types

To analyse differences in adrenocortical gene expression profiles between cattle with different temperaments, the custom Affymetrix® GeneChip® Bovine Gene v1 Array with 10,986 transcripts remaining after filtering was used. The analyses of variance revealed 2,944 genes that differed significantly in their mRNA abundance in at least one comparison between the temperament types.

Overall, the greatest differences were observed between "fearful/neophobic-alert" animals and all other temperament types and fewer differences were observed between the temperament types TT2, TT3 and TT5, as shown by two-way Wald hierarchical clustering of these genes that indicated a distinct differentiation among the gene expression profiles of the different temperament types (Figure 4.1). In this figure, the expression of significant genes is clustered separately for TT1. The expression profiles of TT2 to TT5 showed less distinct differences, but "outgoing/neophilic-alert" (TT3) animals were clustered separately compared to TT2, TT4 and TT5.

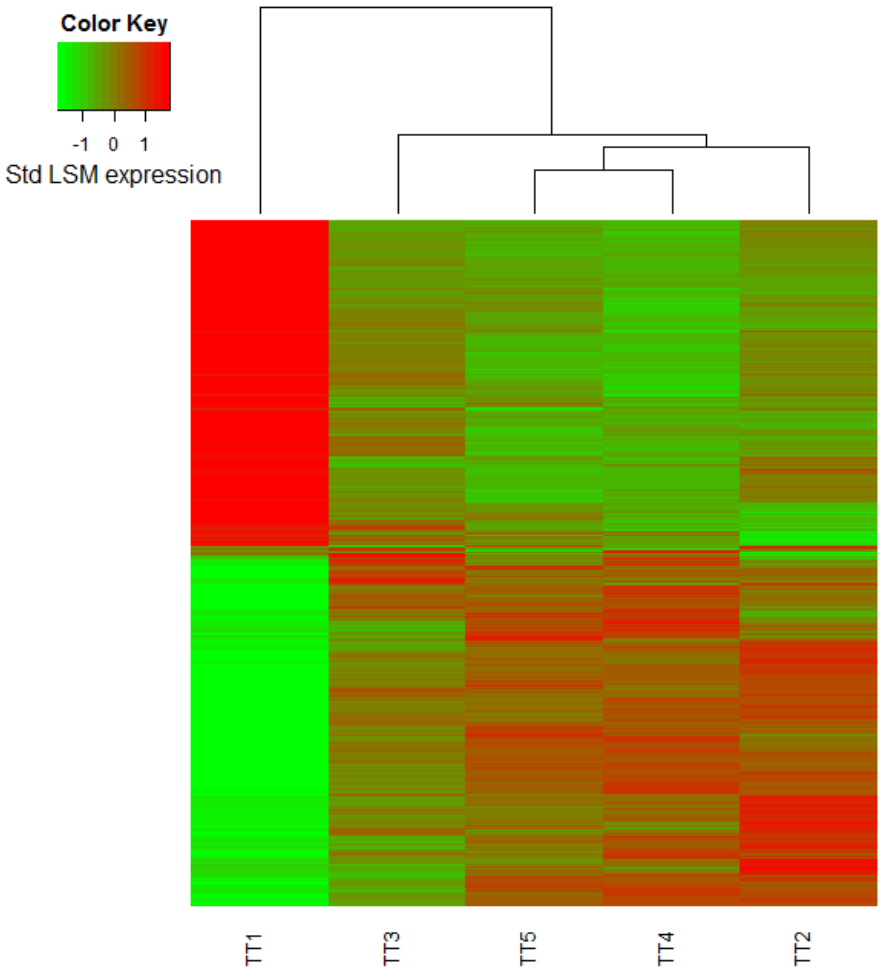

Figure 4.1 Wald hierarchical clustering of standardized LSM expression values of genes significantly differentially expressed among temperament types. Rows are genes and columns are temperament types (TT).

The highest number of significant transcripts was found for the comparison between "fearful/neophobic-alert" animals with TT2 (n = 1329), TT3 (n = 275), TT4 (n = 2002), and TT5 (n = 1830) (Figure 4.2). These comparisons had twelve significantly differentially expressed genes in common. *CCT6A, COPB1, DDX20, DDX52, EE1A1, GALNT1, GMFB, HIF1A, HSF2, SMNDC1* and *THAP5* were down-regulated and *MDK* was up-regulated in comparison to the average gene expression in TT1. Figure 4.2 further illustrates that the greatest differences based on the number of significantly differentially expressed genes were found between TT1 and TT4, followed by the comparison of TT1 with TT5, TT2 and TT3. Therefore, we decided to concentrate on the comparisons between TT1 and all other temperament types and applied a fold change (FC) of 1.5 for the functional characterization of differentially expressed transcripts. The FC was computed as the ratio between normalized expression values of a particular temperament type group.

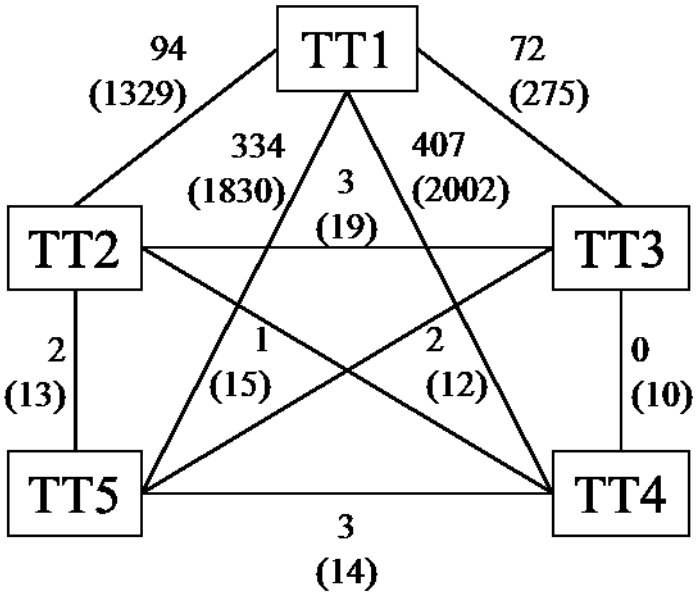

Figure 4.2 Number of significantly differentially expressed genes (adjusted *P*-value < 0.05) between temperament types (TT) filtered for FC ≥ 1.5 and annotation and (without filter criteria).

Pathway analyses

All significantly enriched pathways at *P*-value < 0.05 were mainly involved in physiological stress response and immune function (Table 4.1 and Table S4.2 to S4.5). Different immune-related pathways were observed in all comparisons whereas other pathways were specific for distinct comparisons. The most common canonical pathways were enriched in genes with significant different mRNA abundance between cows classified into TT1 and cows classified into TT2, TT4 and TT5. For these comparisons, the expression of genes involved in 'NRF2-mediated oxidative stress response', in the 'Protein-Ubiquitination Pathway' and 'UDP-N-acetyl-D-galactosamine Biosynthesis II' was significantly lower compared to TT1, although the significant genes differed within pathways. The 'Protein-Ubiquitination pathway' had the highest significance level with –log(*P*-value) = 7.37 in the comparison between TT1 and TT5. Likewise, the expression of genes involved in 'Aldosterone Signalling in Epithelial Cells' was significantly lower in TT2 and TT5 in comparison to TT1, similar to genes that are involved in 'Glucocorticoid Receptor Signalling' and showed a lower expression in TT2 and TT4 in comparison to TT1. Enriched pathways in the comparison of TT3 with TT1 merely overlap with enriched pathways in the other comparisons, which is in accordance with the separate clustering of cows classified in TT3 (Figure 4.1). Enriched pathways of these genes were primarily related to immune function (Table 4.1).

Table 4.1 Top five significantly enriched canonical pathways.

TT	Canonical Pathways	-log(*P*-value)	Ratio
1 vs 2	Aldosterone Signalling in Epithelial Cells	3.76	0.04
	IGF-1 Signalling	3.71	0.05
	NRF2-mediated Oxidative Stress Response	3.48	0.04
	Myc Mediated Apoptosis Signalling	3.46	0.07
	Neurotrophin/TRK Signalling	3.35	0.06
1 vs 3	Complement System	3.56	0.10
	T Helper Cell Differentiation	2.76	0.05
	Crosstalk between Dendritic Cells and Natural Killer Cells	2.67	0.05
	TR/RXR Activation	2.35	0.04
	LPS/IL-1 Mediated Inhibition of RXR Function	2.10	0.02
1 vs 4	Protein Ubiquitination Pathway	3.64	0.07
	Diphthamide Biosynthesis	2.75	0.67
	Assembly of RNA Polymerase II Complex	2.28	0.11
	Telomere Extension by Telomerase	2.27	0.20
	Nucleotide Excision Repair Pathway	2.08	0.12
1 vs 5	Protein Ubiquitination Pathway	7.37	0.09
	NRF2-mediated Oxidative Stress Response	2.86	0.06
	Aldosterone Signalling in Epithelial Cells	2.66	0.06
	CDK5 Signalling	2.07	0.07
	UDP-N-acetyl-D-galactosamine Biosynthesis II	1.89	0.22

Temperament type (TT), name of canonical pathway; -log(*P*-value) of the Fisher`s exact test; ratio of the number of genes from the list that maps to the canonical pathway divided by the total number of genes that map to the same canonical pathway

IPA biological functions that were enriched for genes for which expression was different between temperament types are listed in Table 4.2. In general, terms that were frequently enriched are 'Cell death and survival', 'Cell-to-cell signalling and interaction', 'Cellular movement', 'RNA post-transcriptional modification' and 'DNA replication, recombination and repair'. For significantly differentially expressed genes between TT1 and TT5, the term 'Behaviour' was enriched with *P*-value $=1.98 \times 10^{-2}$ to 3.56×10^{-5}

Table 4.2 Top five significantly enriched biological functions

TT	Diseases and Disorders			Molecular and Cellular Functions			Physiological System Development and Function		
1 vs 2	Name	*P*-value	n	Name	*P*-value	n	Name	*P*-value	n
	Connective Tissue Disorders	$1.51x10^{-5}$	12	Cellular Movement	$9.80x10^{-5}$	15	Connective Tissue Development and Function	$9.80x10^{-5}$	9
	Developmental Disorder	$1.51x10^{-5}$	13	Cellular Growth and Proliferation	$1.36x10^{-4}$	18	Tissue Development	$1.36x10^{-4}$	15
	Hereditary Disorder	$1.51x10^{-5}$	13	Amino Acid Metabolism	$6.76x10^{-4}$	3	Haematological System Development and Function	$8.98x10^{-4}$	12
	Skeletal and Muscular Disorders	$1.51x10^{-5}$	9	Nucleic Acid Metabolism	$6.76x10^{-4}$	9	Haematopoiesis	$8.98x10^{-4}$	8
	Cancer	$2.95x10^{-5}$	85	Small Molecule Biochemistry	$6.76x10^{-4}$	11	Lymphoid Tissue Structure and Development	$8.98x10^{-4}$	7
1 vs 3	Infectious Diseases	$1.12x10^{-6}$	19	Cellular Movement	$5.37x10^{-7}$	27	Immune Cell Trafficking	$5.37x10^{-7}$	24
	Inflammatory Response	$1.32x10^{-6}$	33	Cell-To-Cell Signalling and Interaction	$2.07x10^{-6}$	27	Haematological System Development and Function	$5.82x10^{-7}$	30
	Cardiovascular Disease	$6.30x10^{-6}$	17	Cell Death and Survival	$5.18x10^{-6}$	26	Tissue Development	$6.29x10^{-6}$	18
	Organismal Injury and Abnormalities	$6.30x10^{-6}$	30	Cellular Function and Maintenance	$5.90x10^{-6}$	15	Tissue Morphology	$6.30x10^{-6}$	25
	Developmental Disorder	$3.25x10^{-5}$	15	Protein Synthesis	$3.72x10^{-5}$	9	Connective Tissue Development and Function	$2.23x10^{-5}$	16
1 vs 4	Immunological Disease	$4.32x10^{-6}$	25	RNA Post-Transcriptional Modification	$1.92x10^{-6}$	26	Embryonic Development	$8.58x10^{-5}$	44
	Hereditary Disorder	$2.97x10^{-4}$	53	Cellular Assembly and Organization	$2.60x10^{-4}$	29	Organismal Survival	$8.58x10^{-5}$	96

	Neurological Disease	$2.97x10^{-4}$	43	Cell Death and Survival	$5.19x10^{-4}$	85	Tissue Morphology	$6.04x10^{-4}$	52
	Organismal Injury and Abnormalities	$2.97x10^{-4}$	310	Cell-To-Cell Signalling and Interaction	$6.04x10^{-4}$	24	Organismal Development	$1.11x10^{-3}$	34
	Psychological Disorders	$2.97x10^{-4}$	7	DNA Replication, Recombination, and Repair	$6.18x10^{-4}$	31	Tissue Development	$1.11x10^{-3}$	43
1 vs 5	Cancer	$8.08x10^{-5}$	259	DNA Replication, Recombination, and Repair	$3.52x10^{-6}$	33	Behaviour	$3.56x10^{-5}$	8
	Gastrointestinal Disease	$8.08x10^{-5}$	200	Cell Death and Survival	$4.12x10^{-5}$	55	Embryonic Development	$4.37x10^{-5}$	46
	Organismal Injury and Abnormalities	$8.08x10^{-5}$	263	RNA Post-Transcriptional Modification	$1.62x10^{-4}$	20	Organismal Survival	$4.37x10^{-5}$	81
	Haematological Disease	$1.37x10^{-4}$	65	Cell Cycle	$2.37x10^{-4}$	50	Organismal Development	$5.90x10^{-4}$	38
	Immunological Disease	$1.37x10^{-4}$	41	Cell-To-Cell Signalling and Interaction	$3.90x10^{-4}$	13	Tissue Development	$5.90x10^{-4}$	28

Temperament type (TT), minimum *P*-value of the Fisher`s exact test; number of molecules (n)

Discussion

In the present study, we analysed the bovine adrenocortical transcriptome for the first time and investigated differences in gene expression profiles at slaughter between temperament types assessed in a challenging test situation early in life to gain new insights into the molecular architecture of cattle temperament. We showed that there are differences in gene expression profiles among cows with distinct temperament types involved in molecular pathways that have previously been shown to be involved in the stress or fear response.

Particularly significant differences of gene expression were found for the comparison of "fearful/neophobic-alert" animals and all others. This discrimination becomes clear in the standardized gene expression of significant transcripts (Figure 4.1) and the highest number of significantly differentially expressed genes that were identified for these comparisons (Figure 4.2).This observation probably reflects the role of adrenal cortex in the HPA axis. In contrast, the low differences between the gene expression profiles of TT2, TT3, TT4 and TT5 suggest that temperament features such as 'interested' or 'neophilic' are primarily affected by tissues and organs other than the adrenal cortex, as was shown for prefrontal cortex (Brand *et al.* 2015).

During the pre-slaughtering period which was standardised for all cows, all animals were exposed to emotional stressors; mainly novelty, social separation, and human handling. In cattle, parameters of the HPA axis and SAM activity were shown to differ among individuals in stressful situations (King *et al.* 2006; Curley Jr. *et al.* 2008; Cafe *et al.* 2011a; Burdick *et al.* 2011b). Moreover, adaptive physiological and behavioural response mechanisms to stress were observed to be relatively stable across time and stressful situations in cattle (Müller & Schrader 2005; van Reenen *et al.* 2013) and in other species (Sih *et al.* 2004; Koolhaas *et al.* 2010). On the basis of this current knowledge, our results support the hypothesis of a relatively stable molecular response to stressful situations that is observable as differences in gene expression profiles at slaughter in our study. Moreover, the clear discrimination of "fearful/neophobic-alert" animals is in line with the observation that human handling and presence, which took place in the NH test as well as during transportation and slaughtering, is a strong psychological factor underlying cattle temperament (Adamczyk *et al.* 2013). This indicates that fear could be the main factor responsible for the differences in gene

expression between the "fearful/neophobic-alert" temperament type and all other temperament types at slaughter. Likewise in another study, it was demonstrated that the fear response was more consistent when humans were involved in different behavioural tests performed in beef heifers (Mazurek *et al.* 2011).

The role of 'fear' in the discrimination of temperament types at the molecular level is further confirmed by genes that have been shown to affect fear. For example, the gene *FMR1* was down-regulated in "fearful/neophobic-alert" animals in comparison to the indistinct animals (TT5). *FMR1* knockout mice have been reported to show reduced anxiety (Eadie *et al.* 2009). Additionally, the growth factor *midkine* has been reported to substantially affect the development of hippocampus in knock-out mice (Nakamura *et al.* 1998) as well as in the foetal adrenal in humans and rats (Dewing *et al.* 2000; Ishimoto *et al.* 2006). For *MDK* (-/-) mice, alterations in calcium-binding proteins expression were accompanied by increased anxiety in behavioural tests (Nakamura *et al.* 1998). Similarly, we found that the adrenal expression of *MDK* was lower in the "fearful/neophobic-alert" animals (TT1) in contrast to other temperament types.

The canonical pathway analysis revealed a significant role for immune and stress response mechanisms in the discrimination of temperament types. Thus, genes involved in 'NRF2-mediated oxidative stress response', 'Glucocorticoid Receptor Signalling' or in 'Complement System' and corresponding pathways were significantly differentially expressed. However, differences in enriched pathways and biological functions between the distinct temperament types in the comparison to TT1 were observed. In this study, pathways of the immune system were primarily enriched in genes that were significantly differentially expressed between the "fearful/neophobic-alert" (TT1) and the outgoing/neophilic-alert (TT3) cows. Expression of genes involved in the 'Complement System', 'T Helper Cell Differentiation' and 'Crosstalk between Dendritic Cells and Natural Killer Cells' were up-regulated in TT3 compared to TT1. Because the immune system is one of the defence mechanisms to environmental challenges, stress has been controversially implicated in the effect on the immune status of an organism (Salak-Johnson & McGlone 2007). This result can be seen as a further indication of a suppressive effect of stress hormones such as glucocorticoids on immunity, assuming that TT1 cows were more susceptible to stress. In accordance, differences in the innate immune system and the acute phase response due to

temperament have been observed in cattle and moreover, these differences have also been shown to affect the stress response, indicating a mutual relationship (reviewed in Hughes *et al.* 2014).

Expression profiles of genes within the 'NRF2-mediated oxidative stress response' pathway were different between 'interested/stressed' (TT2), "subdued/uninterested-calm" (TT4) and indistinct (TT5) cows in comparison to "fearful/neophobic-alert" (TT1) cows. Oxidative stress is a disturbance of cellular redox homeostasis caused by physiological or psychological stressors and is assumed to have an effect on steroidogenesis when occurring in the adrenocortical environment (Prasad *et al.* 2014). Moreover, the glucocorticoids themselves have been reported to modulate the onset of oxidative stress (Spiers *et al.* 2014). It can be assumed that cows classified as "fearful/neophobic-alert" were more susceptible to stress during the NH test and slaughtering and thus, the need for antioxidant mechanisms increased in comparison to others or the physiological response mechanisms to stress were differentially regulated, resulting in different oxidative stress levels. Accordingly, the expression of genes enriched in the oxidative stress response was significantly lower in TT2 (*PIK3R3, NRAS, DNAJC3, KRAS, DNAJC10, FKBP5*), TT4 (*CUL3, DNAJC21, NRAS, UBE2K, DNAJC3, DNAJC10, FKBP5, DNAJB14*) and TT5 (*CUL3, DNAJC21, NRAS, DNAJB4, UBE2K, DNAJC3, KRAS, DNAJC10, FKBP5, DNAJB14*) in comparison to TT1. Similarly, Filiou *et al.* (Filiou *et al.* 2011; Filiou *et al.* 2014) revealed an important role for oxidative stress in the biological background of anxiety in mice. In their studies, they analysed metabolites and proteins in cingulate cortex and serum of mice selected for high and low anxiety-related behaviour and showed an increased antioxidant capacity in low-anxiety mice.

'Glucocorticoid Receptor Signalling' was another stress-related mechanism that was significantly enriched in TT1, compared to TT2 and TT4. Because the glucocorticoid receptor mediates the action of cortisol in the target tissues (Bamberger *et al.* 1996) and can directly affect the glucocorticoid level by a negative feedback mechanism, differences in the abundance of genes involved in glucocorticoid receptor signalling further support a temperament type-dependent regulation of the response to stressful situations. Glucocorticoid receptor expression in the adrenal gland may underlie variations in the HPA axis function (Briassoulis *et al.* 2011). Accordingly, cortisol

concentrations were found to be positively associated with excitability and temperament in cattle, indicating variations in the individual stress response in cattle (Grignard *et al.* 2001; King *et al.* 2006; Curley Jr. *et al.* 2008; Burdick *et al.* 2010). In a study by Brand *et al.* (2015) similar results were reported for the expression of brain metabolites in the same experimental cows investigated in this study and the comparison between temperament types. A higher abundance of glucocorticoid 5a-tetrahydrocorticosterone in the "fearful/neophobic-alert" cows was shown in prefrontal cortex, especially in contrast to TT4 and TT5.

The functional analysis of genes with significantly different mRNA abundance among TT1 and the other temperament types generally indicated the importance of adrenal development on cattle temperament. IPA analyses revealed that the functional categories associated with cellular processes such as cell growth, proliferation, signalling and survival were primarily enriched (Table 4.2). Regarding gene expression regulation in the functional categories, no clear trends were observable, for example, the function 'Cell-to-cell signalling and interaction' was primarily up-regulated in the comparison of TT1 and TT3, and primarily down-regulated in the comparisons of TT1 and TT4 as well as of TT1 and TT5. Genes functionally enriched in 'Cell death and survival' were somewhat down-regulated in comparison to TT1. Coincidentally, Muráni *et al.* (2011) found significantly differentially expressed genes involved in mechanisms of cell growth and proliferation among groups of pigs exposed to different levels of psychosocial stress. Genes that were down-regulated in the high-stress pig group were primarily involved in cell death, cellular development, growth and proliferation, terms that we highlighted in our study in the comparison of "fearful/neophobic-alert" animals and all other temperament types. These researchers further assumed a biphasic effect of ACTH on adrenocortical cell growth because growth-stimulating and -inhibiting genes were simultaneously down-regulated in highly stressed pigs.

In recent transcriptome analyses related to temperament in other livestock species such as chickens and pigs, the adrenal response to stress was addressed directly by ACTH treatment and behavioural phenotyping in treated and non-treated groups (Hazard *et al.* 2008; Bureau *et al.* 2009; Muráni *et al.* 2011). As a novelty compared with recent studies, the subjects of this study were grouped according to their temperament in a challenging test situation without consideration of stress levels. However, some

accordance exists between our results and the literature cited above. Several significantly regulated genes were in common, for example, up-regulation of *HSD17B7* (TT1 and TT4) after ACTH treatment in chickens (Bureau *et al.* 2009), or differentially expressed genes between pig breeds that show temperamental differences (*RNF2, MDK, MIF, PTPMT1, CITED2, MDH2,* and others) as well as after ACTH treatment (*TOMM20, DDX3X, GNL2, WASF2*, and others) (Hazard *et al.* 2008), but the FC of these genes was less than 1.5. Furthermore, the enriched biological functions and canonical pathways by IPA correspond well with those obtained in the study of Muráni *et al.* (2011). In that study, adrenal transcriptome of pigs that were exposed to various levels of psychological stress were compared. The 'Protein Ubiquitination Pathway', as well as 'NRF2-mediated Oxidative Stress Response' were enriched in up-regulated genes and 'IGF-1 Signalling' in the down-regulated genes in the high-stress group. The 'Protein Ubiquitination Pathway' and 'NRF2-mediated Oxidative Stress Response' were significantly down-regulated in "interested-stressed" (TT2) and indistinct (TT5) animals in comparison to TT1. In contrast, 'IGF-1 Signalling' was down-regulated in TT2 compared to TT1. According to the observations of Muráni *et al.* (2011), the "fearful/neophobic-alert" animals in this study could correspond to the high-stress pig group in their study.

Conclusions

This study provided new insights into adrenal molecular differences in cattle that had different temperament types in a novel-human test. Several biological functions of the differentially expressed genes between the "fearful/neophobic-alert" cows and the others have been described in earlier reports concerning the adrenal stress response in livestock species, for example, immune function and stress response pathways, which were down and up regulated, respectively, especially in comparison to "subdued/uninterested-calm" cows. An additional pivotal role in the association of cattle temperament and adrenal expression profiles could be assigned to genes that are involved in cellular processes. The clear distinction of "fearful/neophobic-alert" cows from the expression profiles of all other cows indicates a prominent role for fearfulness in behaviour manifestation even at the molecular level. Our results further support the assumption of consistency in the individual behavioural responses in challenging

situations at the molecular level because we identified relationships between differences in adrenal gene expression at slaughter during the second lactation and temperament types assigned to the calves early in life. In conclusion, this study provides new targets for further research on cattle temperament; nevertheless, further work is required to elucidate the role of the potential molecular targets proposed in this study.

Acknowledgements

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Chapter 5 Additional analyses

Identification of adrenocortical transcripts correlated with behavioural traits and GWAS for temperament types

In Chapter 2, phenotyping cattle temperament is described as crucial step in the biological analysis of cattle behaviour, because a wide variety of approaches exists (reviewed in Canario *et al.* 2013). A distinction can be drawn between procedures based on test situations or in-field observations and between phenotypes based on objective measurements, for example flight distance, or subjective temperament assessments as described in Grandin (1993). According to the numerous possibilities to phenotype cattle behaviour, the consequences of using behavioural traits or temperament types in the analyses carried out in this thesis should be investigated. Therefore, the behavioural traits (DA, DI, DE in OF and NO test) were correlated to adrenocortical gene expression and the association between genome-wide SNPs and temperament types was analysed in a GWAS.

Of the 147 cows with behavioural trait records (DA, DI, DE in OF and NO test) described in Chapter 3, adrenocortical gene expression profiles were available for 111 cows. RNA isolation, gene expression profiling and data processing are described in Chapter 4. For these 111 cows, Spearman rank correlation was analysed between the expression of 10,986 genes and the particular behavioural trait using the cross correlation tool in JMP Genomics 5.1. To account for multiple testing, the FDR adjustment was applied for *P*-value = 0.05.

A total of 82 cows had genotype data and were assigned to the five temperament types in NH test (described in Chapter 4). DNA isolation, genotyping and data processing are described in Chapter 3. For these cows, a GWAS was performed to identify associations between 37,201 single SNPs and temperament types. GWAS was done using the SNP-trait association tool in JMP Genomics 5.1. In this analysis, no fixed effects were implemented, in contrast to the GWAS described in Chapter 3, because of the small sample size and the resulting heterogeneous animal numbers in the particular groups that can promote the occurrence of false positives. To account for multiple testing, a Bonferroni correction was applied for *P*-value = 0.05 at a genome-wide and a chromosome-wide significance level.

As a results of the correlation testing between behavioural traits DA, DI and DE recorded in OF and NO test and adrenocortical gene expression profiles, nine adrenocortical transcripts were identified to be significantly correlated to DA in OF test with FDR-adjusted *P*-value < 0.05 (Table 5.1). For the traits DI and DE in OF as well as for DA, DI and DE in NO test, no significant genes were found with this significance threshold.

Table 5.1 Adrenocortical transcripts significantly (FDR-adjusted *P*-value < 0.05) correlated with duration of active behaviour in open-field test

Transcript	**r_s**	***P*-value**	**Chr**	**start**	**stop**	**Gene**
12804914	-0.39	2.15×10^{-7}	23	31525879	31526272	*LOC505183*
12802575	-0.40	1.38×10^{-5}	23	31479375	31479686	*LOC527388*
12803480	-0.40	1.33×10^{-5}	23	31526564	31527042	*LOC613926*
12806857	-0.40	1.17×10^{-5}	23	31607312	31607790	*LOC617905*
12847425	-0.40	0.10×10^{-5}	3	20803166	20803930	*H2B*
12836963	-0.40	1.05×10^{-5}	3	20793546	20793962	*HIST2H2BE*
12841208	-0.43	3.08×10^{-6}	3	20825192	20825680	*HIST2H2BF*
12804010	-0.43	2.58×10^{-6}	23	31519198	31519765	*H2B*
12805856	-0.46	3.88×10^{-7}	23	31472290	31472758	*LOC521580*

FDR = false discovery rate; r_s = correlation coefficient; Chr = chromosome

In the GWAS for the classification into temperament types in NH test, no SNPs were significantly associated with temperament types at a genome-wide and chromosome-wide Bonferroni corrected *P*-value of $P\text{-value}_{genome} = 1.34 \times 10^{-6}$ and $P\text{-value}_{chromosome} = 1.08 \times 10^{-4}$ to $P\text{-value}_{chromosome} = 2.15 \times 10^{-5}$ (Figure 5.1). The SNP "rs444483442" on BTA24 reached the minimum *P*-value of 0.0006.

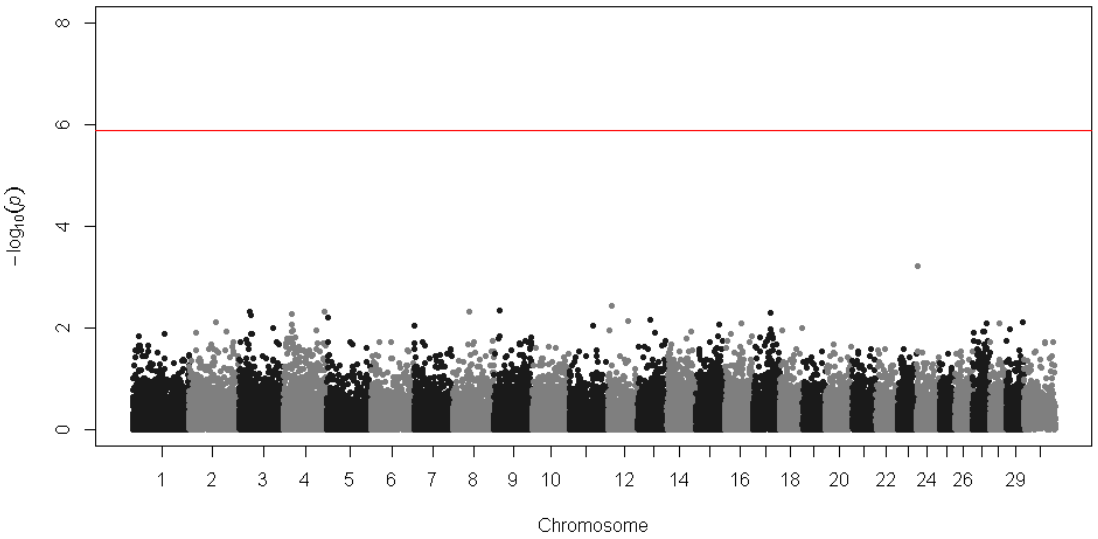

Figure 5.1 Manhattan plot for the GWAS between 37,201 SNPs and temperament types of NH test. Red line represents the genome-wide significance threshold of *P*-value = 1.34×10^{-6}

Relationship between milk performance traits and temperament types

In literature, a relationship between cattle temperament and milk performance is described. Especially in challenging situations, a suppressive effect of stress on milk yield was observed (Breuer *et al.* 2000; Hemsworth *et al.* 2000; Sutherland *et al.* 2012). To analyse the relationship between temperament types and milk performance traits in this thesis, an ANOVA was applied for the temperament types of the 60 cows described in Chapter 4 and their corresponding milk performance traits MY, yield5, yield30, R1 and R3 in the first lactation. For comparing the group means of the particular temperament types, a Student's t-test was applied. All analyses were performed using JMP Genomics 5.1 software.

The ANOVA for the influence of temperament types assessed in NH test on milk performance traits revealed a significant effect of temperament types on the milk yield day 1 to 5 (yield5, *P*-value = 0.02) and day 6 to 30 (yield30, *P*-value = 0.04) of lactation. The comparison of milk performance traits between temperament types using Student's t-test showed a substantial discrimination of "fearful/neophobic-alert" cows

from others regarding milk performance traits (Figure 5.2). These cows had on average significant lower milk yields and the drop in milk performance after rehousing was more pronounced. “Outgoing/neophilic-alert” (TT3) cows had the highest average milk yield (MY) and the highest milk yield day 1 to 5 and day 6 to 30 of lactation. This difference was significant in comparison to TT1, but not to the other temperament types. The drop in milk yield after rehousing was significantly higher for “fearful/neophobic-alert” cows (TT1) in regard to “outgoing/neophilic-alert” ones (TT3) with R1 = 1.56 for TT1 as well as 1.13 for TT3 and R3 = 2.00 for TT1 as well as 1.29 for TT3. Cows assigned to be “interested-stressed” (TT2) had the lowest drops in milk yield after rehousing.

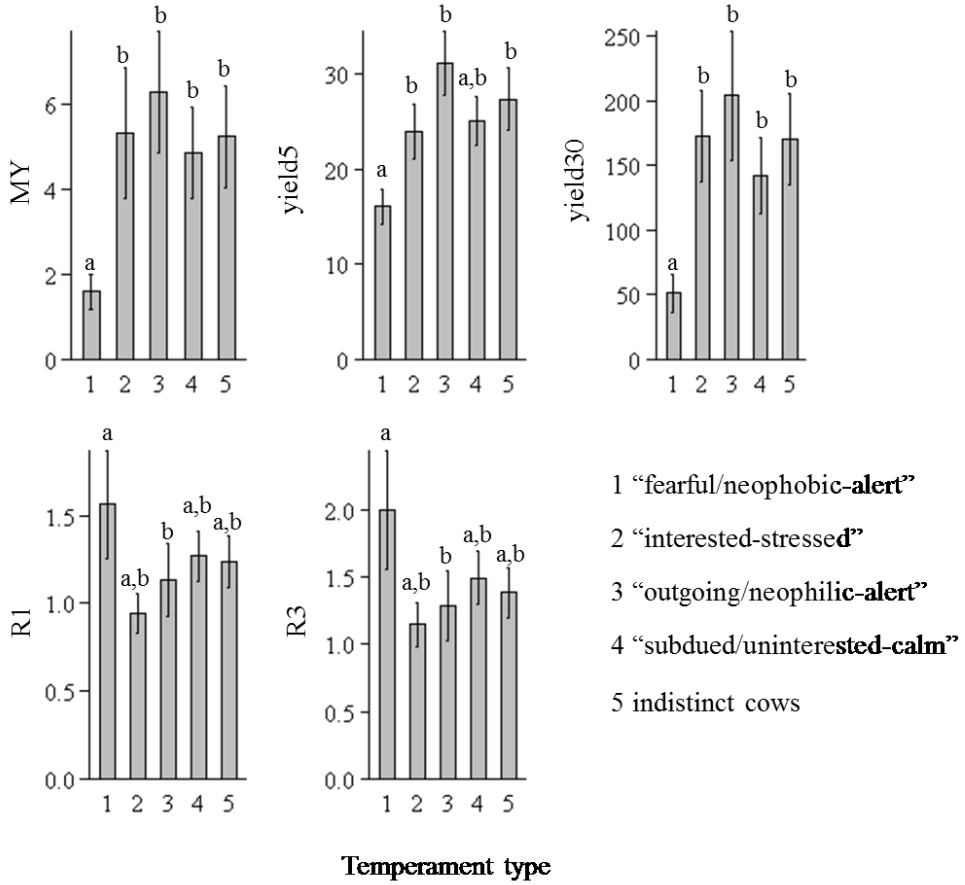

Figure 5.2 Boxplots for temperament type effect on milk performance parameters average daily milk yield (MY; kg), milk yield day1 to 5 (yield5; kg), milk yield day 6 to 30 (yield30; kg), ratio of milk yield one day before and after rehousing (R1) and three days before and after rehousing (R3). Different letters represent significant differences for *P*-value = 0.05

Identification of adrenocortical transcripts correlated with milk performance traits

The adrenal cortex is suggested as target tissue for behavioural studies, especially in regard to stress responsiveness (Mormède *et al.* 2007; Mormède *et al.* 2011). Additionally, glucocorticoids synthesized in the adrenal cortex were shown to be increased on the day of parturition indicating a role in lactation initiation (Akers *et al.* 1981) and further contribute to energy supply during lactation by determining lipolysis in adipose tissue (Lanna & Bauman 1999). To investigate the adrenal cortex as possible mutual target tissue of cattle behaviour and milk performance, adrenocortical gene expression was correlated to milk performance traits as supplementation to the results of Chapter 4. Of the 147 genotyped cows (Chapter 3), adrenocortical gene expression profiles (Chapter 4) and milk performance data were available for 111 cows. RNA isolation, gene expression profiling and data processing are described in Chapter 4. For these 111 cows, Spearman rank correlation was analysed between the expression of 10,986 genes and the particular milk performance traits MY, yield5, yield30 and R1 of the second lactation using the cross correlation tool in JMP Genomics 5.1. To account for multiple testing, the FDR adjustment was applied for *P*-value = 0.05. The functional analysis for significant transcripts was performed using IPA. Both, *P*-values for the enrichment of canonical pathways and biological functions of the significant genes, were corrected using an FDR-adjusted *P*-value of 0.05.

For the association of milk performance traits with adrenocortical gene expression, 228 significant correlations were identified for milk performance at the beginning of lactation (yield5), 31 for the average milk yield (MY), 3 for milk performance from day 6 to 30 of lactation and none for the response to rehousing (R1) in the second lactation. Correlation coefficients ranged from -0.43 to 0.42 for yield5, -0.53 to 0.50 for yield30 and -0.50 to 0.48 for MY (Table S5.1). Transcripts significantly correlated with yield5 were enriched in “EIF2 signaling” canonical pathway (FDR-adjusted *P*-value = 0.01; *PAIP1*, *RPL6*, *RPL10*, *RPL17*, *RPLP1*, *RPS17*, and *RPS27*) and have main functions in protein synthesis (FDR-adjusted *P*-value = 0.03; *EEF1A1*, *IGF2*, *ILF3*, *PAIP1*, *PPARA*, *RHEB*, *RPL17*, *RPS17*, *RPS27L*, *SUMO1*). Of the 31 transcripts significantly correlated with MY, *PDGFRA*, *PDGFRB* and *PPARA* are involved in “PPAR Signaling” canonical pathway (FDR-adjusted *P*-value = 0.02) and in general, these 31 transcripts have functions in lipid metabolism and energy production (Table S5.2).

Chapter 6 General discussion

6.1 Genetic variations and molecular mechanisms associated with cattle behaviour

One of the upcoming major questions in cattle farming is the selection for cattle behaviour and its potential consequences on production traits. To address the issue of cattle behaviour and underlying genetic and molecular mechanisms, especially in regard to milk performance, two strategic approaches were applied: the identification of genetic variations affecting behavioural traits of locomotion and exploration by performing a GWAS (Chapter 3) and revealing adrenal transcriptome alterations associated with temperament types in a challenging situation (Chapter 4). The objective of this study was to make a contribution to breeding for cattle temperament by giving initial indications of putative biomarkers for cattle behaviour and investigating the molecular interplay between behaviour and milk performance.

GWAS revealed genetic regions associated with behavioural traits

In general, behaviour is assumed to be influenced by environment, experiences and genetic prerequisites (Mormède *et al.* 2002; Mormède 2005). A confirmation for the latter factor is demonstrated by the results presented in Chapter 3, which underpin the idea of a genetic background of cattle behaviour. We were able to identify several SNPs that were significantly associated with activity, inactivity and exploration in OF and NO test in a GWAS. Previous studies on the genetics of cattle behaviour or temperament primarily identified QTL by using microsatellite markers (Wegenhoft 2005; Gutiérrez-Gil *et al.* 2008; Boldt 2008; Glenske *et al.* 2011). Besides this methodical difference, multiple genomic regions found during the analyses in this thesis were similar to results of these studies, as illustrated in Table 6.1. For ease of comparison, we assumed that the average genetic distance per Mbp is 1.25 according to Arias *et al.* (2009). Table 6.1 further demonstrates the complexity of genetic influence on behaviour by numerous significant associations that were distributed across almost all chromosomes. The complexity of behaviour in general, affected by environment and individual experiences besides a genetic background as discussed in Chapter 2, make the major challenge in behavioural genetics not the detection of QTL, but rather the identification of causal genes (Andersson & Georges 2004). For genomic selection, the identification of genetic markers, which have a preferably additive genetic effect size, is of interest, which

means that a sufficient large proportion of the phenotypic variance should be attributable to the additive genetic effect. In recent livestock breeding, genetic markers are available for causal mutations of single-gene traits, for example diseases, rather than complex traits (Goddard & Hayes 2009). In contrast, behaviour is assumed to be under the control of numerous genes (Jensen 2006) and the proportion of genetic influences on variability between individuals is assumed to be merely 10% (Flint 2003). In accordance to these hypotheses, several genetic loci significantly associated to behavioural traits were identified in Chapter 3 that explained only a small amount of phenotypic variability.

Furthermore, although there are some neighbouring or overlapping genetic regions associated with behavioural traits, behavioural phenotypes used in the particular studies are heterogeneous. Likewise in this thesis, the behavioural phenotype under investigation seemed to have a profound effect on the results. Only two of the 41 SNPs were significantly associated with more than one behavioural trait and no significant genetic loci overlapped between the different test situations leading to the conclusion that SNPs affecting behaviour are specific for trait and test. Accordingly, Réale *et al.* (2007) described that different behaviour test conditions might trigger particular behavioural characteristics, for example, exploration in OF testing and boldness in NO testing. However, these behavioural characteristics can be phenotypically correlated, as also shown in Chapter 3, especially for the traits activity and inactivity.

This thesis could provide – in combination with information and findings from literature (Table 6.1) – more evidence on a pivotal role of BTA29 on cattle behaviour. The dopamine D4 receptor (*DRD4*) gene located on BTA29 (Larkin *et al.* 2003; Haegeman *et al.* 2003) was recently investigated as functional candidate gene in order to identify associations between genetic variation and beef cattle behaviour (Glenske *et al.* 2011). Currently, *DRD4* is assigned to the position of 50779411 bp to 50781672 bp according to the NCBI Bos taurus Annotation Release 104 (http://www.ncbi.nlm.nih.gov/gene/101906668). In this thesis, we detected three SNPs which were significantly associated with exploratory behaviour in the novel-object test that were assigned to a LD block spanning 418,005 bp (Chapter 3). Additionally, in a QTL mapping study for milking temperament, the tyrosinase gene (*TYR*) on the centre of BTA29 came into focus (Hiendleder *et al.* 2003).

Table 6.1 Quantitative trait loci for cattle behavioural characteristics from literature (adapted from Adamczyk *et al.* (2013)) compared to significant SNPs of Chapter 3

Chr	Trait	Marker	Position (cM)	Breed	Reference
1	habituation+temperament	BMS574	15,42	beef breed cattle from ET	Schmutz *et al*. (2001)
	temperament	DIK70-PIT17B7	37	*Bos taurus* (Angus) x *Bos indicus* (Brahman, Nellore)	Wegenhoft (2005)
	habituation	BM6438	1,78	Charolais x Holstein-Friesian	Gutierrez-Gil *et al*. (2008)
		BMS4044	141		
2	inactivity OF	rs109193448	49,96	Charolais x Holstein-Friesian	Friedrich *et al.* (2015)
		rs111021714	50,31		
		rs43332694	55,83		
3	exploration NO	BTA-122016-no-rs	31,05	Charolais x Holstein-Friesian	Friedrich *et al.* (2015)
	temperament	BM7225-ILSTS64	45	*Bos taurus* (Angus) x *Bos indicus* (Brahman, Nellore)	Wegenhoft (2005)
4	exploration NO	rs29027498	21,20	Charolais x Holstein-Friesian	Friedrich *et al*. (2015)
	temperament	TEXAN17-MAF50	28-51	*Bos taurus* (Angus) x *Bos indicus* (Brahman, Nellore)	Wegenhoft (2005)
	habituation+temperament	MAF50-DIKO26	51,21-86,23	Charolais x Holstein-Friesian	Gutierrez-Gil *et al.* (2008)
5	habituation+temperament	RM103	29,42	beef breed cattle from ET	Schmutz *et al*. (2001)
6	habituation	DIK5076-BM1329	4,51-35,39	Charolais x Holstein-Friesian	Gutierrez-Gil *et al*. (2008)
	temperament	CSSM22-CSM34	1	*Bos taurus* (Angus) x *Bos indicus* (Brahman, Nellore)	Wegenhoft (2005)
	exploration OF	rs41255467	55,35	Charolais x Holstein-Friesian	Friedrich *et al.* (2015)
7	habituation	RM006-BM1853	25,39-85,32	Charolais x Holstein-Friesian	Gutierrez-Gil *et al.* (2008)
	activity NO	rs43708473	89,71	Charolais x Holstein-Friesian	Friedrich *et al*. (2015)
8	temperament	BMS1864-BM3419	0	*Bos taurus* (Angus) x *Bos indicus* (Brahman, Nellore)	Wegenhoft (2005)
	habituation	CSSM047	115,2	Charolais x Holstein-Friesian	Gutierrez-Gil *et al*. (2008)
	activity OF	rs109064778	78.83	Charolais x Holstein-Friesian	Friedrich *et al*. (2015)
9	habituation+temperament	ILSTS013	48,73	beef breed cattle from ET	Schmutz *et al*. (2001)
	temperament	BM6436-BM4208	72	*Bos taurus* (Angus) x *Bos indicus* (Brahman,	Wegenhoft (2005)

				Nellore)	
	temperament	BM2504-UWCA9	30,92-49,99	Charolais x Holstein-Friesian	Gutierrez-Gil *et al*. (2008)
	habituation	BM888-CSRM60	59,98-77,81		
	exploration NO	rs109313646	68,00	Charolais x Holstein-Friesian	Friedrich *et al.* (2015)
		rs111019360	75,75		
10	inactivity OF	rs110260889	7,47	Charolais x Holstein-Friesian	Friedrich *et al*. (2015)
	habituation	BMS528-TGLA378	24,01-43,65	Charolais x Holstein-Friesian	Gutierrez-Gil *et al*. (2008)
	activity NO	rs110025880	16,01	Charolais x Holstein-Friesian	Friedrich *et al.* (2015)
	exploration NO	rs41256789	37,05		
		rs109741931	37,24		
	inactivity NO exploration NO	rs42838073	37,33		
	exploration NO	Hapmap31150-BTA-152385	53,35		
11	habituation+temperament	LISTS036	61,57	beef breed cattle from ET	Schmutz *et al*. (2001)
	habituation	ILSTS100-IDVGA-3	59,11-81,8	Charolais x Holstein-Friesian	Gutierrez-Gil *et al*. (2008)
12	temperament	BMS2252-RM094	20 I 22	*Bos taurus* (Angus) x *Bos indicus* (Brahman, Nellore)	Boldt (2008)
	inactivity NO	Hapmap48681-BTA-19661	26,98	Charolais x Holstein-Friesian	Friedrich *et al.* (2015)
14	inactivity OF	rs108979436	7,19	Charolais x Holstein-Friesian	Friedrich *et al*. (2015)
	habituation+temperament	RM180-ILSTS008	33,31-50,91	beef breed cattle from ET	Schmutz *et al*. (2001)
	inactivity NO exploration NO	rs109784719	35,95	Charolais x Holstein-Friesian	Friedrich *et al*. (2015)
	inactivity NO	rs110245129	54,88		
		rs41666787	56,80		
15	habituation+temperament	ADCY2	22,67	beef breed cattle from ET	Schmutz *et al*. (2001)
	exploration NO	rs109494085	42,60	Charolais x Holstein-Friesian	Friedrich *et al.* (2015)
16	exploration NO	rs109513733	40,64	Charolais x Holstein-Friesian	Friedrich *et al.* (2015)
	temperament	INRA013-BMS462	79	*Bos taurus* (Angus) x *Bos indicus* (Brahman, Nellore)	Wegenhoft (2005)

	temperament	INRA48-BM3509	70	*Bos taurus* (Angus) x *Bos indicus* (Brahman, Nellore)	Boldt (2008)
	temperament	HUJ625	100.2	Charolais x Holstein-Friesian	Gutierrez-Gil *et al.* (2008)
		ETH11-BM719	54.07-77.57		
	habituation	BM121	26.4		
18	temperament	BL1016-BM8151	18	*Bos taurus* (Angus) x *Bos indicus* (Brahman, Nellore)	Wegenhoft (2005)
		IDVGA-31-ABS013	0-15.75	Charolais x Holstein-Friesian	Gutierrez-Gil *et al*. (2008)
19	temperament	CSSM065-ETH3	69.83-90.04	Charolais x Holstein-Friesian	Gutierrez-Gil *et al.* (2008)
	habituation	BMS2142-CSSM065	43.31-69.83		
	inactivity OF	rs29019596	30,53	Charolais x Holstein-Friesian	Friedrich *et al.* (2015)
	exploration NO	rs17597495	14,08		
		Hapmap38959-BTA-44727	18,29		
	inactivity NO	rs110894302	17,72		
20	temperament	DIK015-BM5004	52.49-71.80	Charolais x Holstein-Friesian	Gutierrez-Gil *et al.* (2008)
21	activity OF	BTA-12468-no-rs	7,50	Charolais x Holstein-Friesian	Friedrich *et al.* (2015)
		rs110780905	7,91		
	habituation	HEL10-TGLA337	65	Charolais x Holstein-Friesian	Gutierrez-Gil *et al*. (2008)
22	inactivity OF	rs109674592	41,70	Charolais x Holstein-Friesian	Friedrich *et al.* (2015)
		rs110027993	42,26		
24	inactivity OF	rs29012505	0,74	Charolais x Holstein-Friesian	Friedrich *et al*. (2015)
		Hapmap47669-BTA-59022	0,86		
	activity OF	rs109679723	17,32		
25	activity NO	rs109243151	8,24	Charolais x Holstein-Friesian	Friedrich *et al*. (2015)
	temperament	BM737-INRA222	31.59-53.37	Charolais x Holstein-Friesian	Gutierrez-Gil *et al.* (2008)
26	temperament	ABS012-HEL11	9.9	Charolais x Holstein-Friesian	Gutierrez-Gil *et al.* (2008)
		IDVGA59-HEL11	33	*Bos taurus* (Angus) x *Bos indicus* (Brahman, Nellore)	Boldt (2008)

	inactivity NO	rs110898125	38,87	Charolais x Holstein-Friesian	Friedrich *et al.* (2015)
	activity NO				
28	temperament	BP23	10.89	Charolais x Holstein-Friesian	Gutierrez-Gil *et al*. (2008)
	activity NO	rs42138859	19,80	Charolais x Holstein-Friesian	Friedrich *et al.* (2015)
29	milking temperament	BMS764-BMC8012	11.29-21.11	Holstein-Friesian cows	Hiendleder *et al*. (2003)
	exploration NO	rs108965864	15,39	Charolais x Holstein-Friesian	Friedrich *et al*. (2015)
		rs42169108	15,47		
		rs43099931	15,50		
	temperament	DIK094-MNB101	40.16-69.73	*Bos taurus* (Angus) x *Bos indicus* (Brahman, Nellore)	Boldt (2008)
		BMC3224-BMS764	21		
	habituation	RM044-MNB166	24.48-33.51	Charolais x Holstein-Friesian	Gutierrez-Gil *et al.* (2008)
X	activity NO	rs29025765	12,76	Charolais x Holstein-Friesian	Friedrich *et al.* (2015)

Chr = chromosome; cM = centi Morgan; OF = open-field test; NO = novel-object test

Adrenocortical gene expression at slaughter showed differences between temperament types

Genetic studies can hardly capture physiological alterations underlying differences in behavioural traits due to the complexity of the particular mechanisms, but gene expression profiling can provide additional information (Carter *et al.* 2001). In this thesis, the analysis of the adrenocortical gene expression at slaughter in regard to temperament types (TT), to which the cows were assigned early in age, revealed 2,944 transcripts with significant different RNA abundance between at least two of the five analysed temperaments types (Chapter 4).

We primarily assumed an enrichment of stress response pathways by these significant genes as it was previously shown in other adrenocortical transcriptome studies in livestock species (Hazard *et al.* 2008; Bureau *et al.* 2009; Muráni *et al.* 2011), because the adrenal cortex is involved in the physiological stress response as a part of the HPA axis (Mormède *et al.* 2007) and NH test as well as slaughter represent challenging situations. Indeed, it turned out that 'Glucocorticoid Receptor Signalling' pathway was enriched by the comparison between "fearful/neophobic-alert" (TT1) with "subdued/uninterested-calm" (TT4) and "interested/stressed" (TT2) cows as well as the 'NRF2-mediated oxidative stress response' pathway for "interested/stressed" (TT2) and "indistinct" (TT5) cows again in comparison to "neophobic/fearful-alert" ones. The results further reveal the outstanding role of adrenocortical expression profiles of "fearful/neophobic-alert" animals, similar to the observations of Brand *et al*. (2015). In their study, brain and serum metabolite profiles where compared between a subset of the same SEGFAM F2 cows analysed in this thesis. They observed that the cows could be classified into the temperament types according to their metabolite profiles, especially cows grouped in TT1. This is particularly interesting considering that the classification into temperament types was done in early age and tissue sampling for gene expression profiling in the second lactation at slaughter. Accordingly, Boissy (2005) reviewed that fear-related behavioural responses appear to be stable over situations as well as over time and behaviour differences become particularly obvious when fear of humans is addressed in behaviour tests (Mazurek *et al.* 2011). In this thesis, additional measurements, e.g. temperament assessments and cortisol concentration in blood during slaughter, could have been utilised to produce more reliable results for the assumption of consistent behaviour responses. Furthermore, assuming that the "fearful/neophobic-alert" cows are more susceptible to stress throughout time and situations, there should

be associations between temperament types of NH testing and adrenal size and weight, because it was shown that chronic stress resulted in weight loss and increased size of the adrenal gland in rodents and pigs (Mormede *et al.* 1990; Kanitz *et al.* 2005).

Kommadath *et al.* (2011) performed a gene expression profiling in four brain areas to identify transcripts associated with cattle estrous behaviour. Concerning cattle temperament in challenging situations, no studies exist on the relationship between adrenocortical gene expression and cattle temperament. So far, correlations between cattle temperament and adrenal stress hormones quantified in blood plasma, for example, cortisol and epinephrine, were reported (Burdick *et al.* 2011a). The experiment in Chapter 4 gave insights into adrenal mechanisms that might be involved in the expression of behaviour variances between cows that lead to different temperament types. Thus, the role of adrenal cortex in stress and fear related temperament was highlighted by the findings.

Biological background of cattle behaviour is specific for phenotype

To include cattle behaviour in breeding indexes, measurements of behavioural phenotypes have to be standardized. The selection of suitable behavioural phenotypes is not trivial as shown by further results of this thesis that should be discussed in the following.

In the analysis of behavioural traits at the genome-level, 41 significant SNPs were found for the behavioural traits DA, DI and DE recorded in OF and NO test (Chapter 3). At the transcriptome-level, only nine adrenocortical transcripts were identified to be significantly correlated to the duration of active behaviour in the OF test with FDR-adjusted P-value < 0.05 (Chapter 5). The expression of these nine genes was moderately negatively correlated with DA in OF test. Interestingly, they have functions in histone modification. In coincidence, epigenetic programming such as histone modification is discussed to affect HPA axis function (Anacker *et al.* 2014). In contrast in the analysis of temperament types several genes were identified to be significantly differentially expressed (Chapter 4), but no significant SNP was detected for the discrimination of temperament types (Chapter 5).

Presumably, the summarization of behavioural traits into temperament types through PCA leads to a loss of association at the genetic level. This is further emphasized by the main finding that significant genetic loci identified in Chapter 3 were specific for

behavioural trait and test situation. Regarding the comparison at the transcriptome level, one may speculate that temperament types, which are based on a PCA of the recorded behavioural traits, are more robust and thus, consistent across time and test situations. This might have enabled the detection of adrenocortical gene expression differences even within time distance in contrast to the behavioural traits recorded under test conditions.

The analysis of interesting genes identified in the GWAS (Chapter 3) at the transcriptome level could provide further information based on the idea of system biology. Of the 41 SNPs that were significantly associated with the three behavioural traits in OF and NO test, 19 are located in annotated genes. Of these, 8 genes were also represented on the microarray used for analyses in Chapter 4. However, as a result, no direct overlaps between genes with an SNP significant for behavioural traits and significantly differentially expressed genes revealed for temperament types (Chapter 4) or DA in OF test (Chapter 5) could be identified. Furthermore, the nine adrenocortical transcripts significant for DA in OF (Chapter 5) do not overlap with transcripts significantly differentially expressed between temperament types (Chapter 4). This is not surprising considering that first, different behavioural phenotypes measured in different test situations were examined in the particular experiments. The adaption of the same behavioural phenotype in both experiments could have solved this problem, but as discussed above, there would have been no results for one of the analyses, dependent on the selected phenotype. Second, the transcriptome is tissue-specific in contrast to the genome and only one of the putative target tissues for behaviour was under investigation in this thesis considering the adrenal cortex. And third, the heterogeneous sample size for cows assigned to temperament types in NH and to the particular behavioural traits could have influenced the results.

6.2 Behaviour and milk performance

Associations between behavioural phenotypes and milk performance traits

As reported in Chapter 3, there were only significant correlations between behaviour and milk performance traits for the duration of exploratory behaviour in the novel-

object test. Similarly, Orbán *et al*. (2011) could not found correlations between temperament scores of Jersey and Holstein Friesian cows and daily milk yield. Indeed, significant correlations were calculated for the ratio of milk yield before and after rehousing within one (R1) and three (R3) lactation days, which were assumed to indicate the reactivity towards novelty, and milk parameters. Similarly, other authors describe a drop in milk yield after challenging events (Sutherland *et al.* 2012).

An additional analysis of milk performance traits (Chapter 3) concerning the classification into temperament types (Chapter 4) was performed using an ANOVA. This analysis revealed a significant effect of temperament types on the milk yield day 1 to 5 (yield5, *P*-value = 0.02) and day 6 to 30 (yield30, *P*-value = 0.04). Comparisons between temperament types using Student's t-test showed a substantial discrimination of "fearful/neophobic-alert" cows from others regarding milk performance traits (Figure 5.1). These cows had on average significantly lower milk yields and the drop in milk performance after rehousing was more pronounced. This observation again supports the hypothesis that anxiety limits milk production due to stress suppressing the release of oxytocin hormone or its hormone effects, resulting in a disruption of milk removal (Bruckmaier & Blum 1998; van Reenen *et al.* 2002). Especially the drop in milk yield after rehousing in almost every temperament type, except for TT2 in R1, is in accordance with literature that milking in novel environments was observed to affect milk performance negatively, but the results in this thesis also indicate that temperamental differences have an influence on the amount of the response. Thus, the drop in milk yield after rehousing was significantly higher for "fearful/neophobic-alert" cows (TT1) in comparison to "outgoing/neophilic-alert" cows (TT3) with R1 = 1.56 for TT1 and 1.13 for TT3 as well as R3 = 2.00 for TT1 and 1.29 for TT3. Moreover, it is interesting that cows assigned to be "interested-stressed" (TT2) have the lowest drops in milk yield after rehousing. Both, TT2 and TT3 have high PC2 scores which characterise inactivity and exploratory behaviour towards the open-field. This indicates that these cows might have a more positive emotional evaluation when in novel surroundings.

Associations between behavioural phenotypes and milk performance traits with genetic variations and expression profiles

Results of the GWAS indicate a competitive genotype effect for activity and exploratory behaviour in comparison to milk yield and reactivity to rehousing (Chapter 3). This relationship is depicted in Figure 6.1 the three significant SNPs on BTA29 assigned to a linkage block that were significant for DE in NO and milk performance traits (MY, yield30, R3).

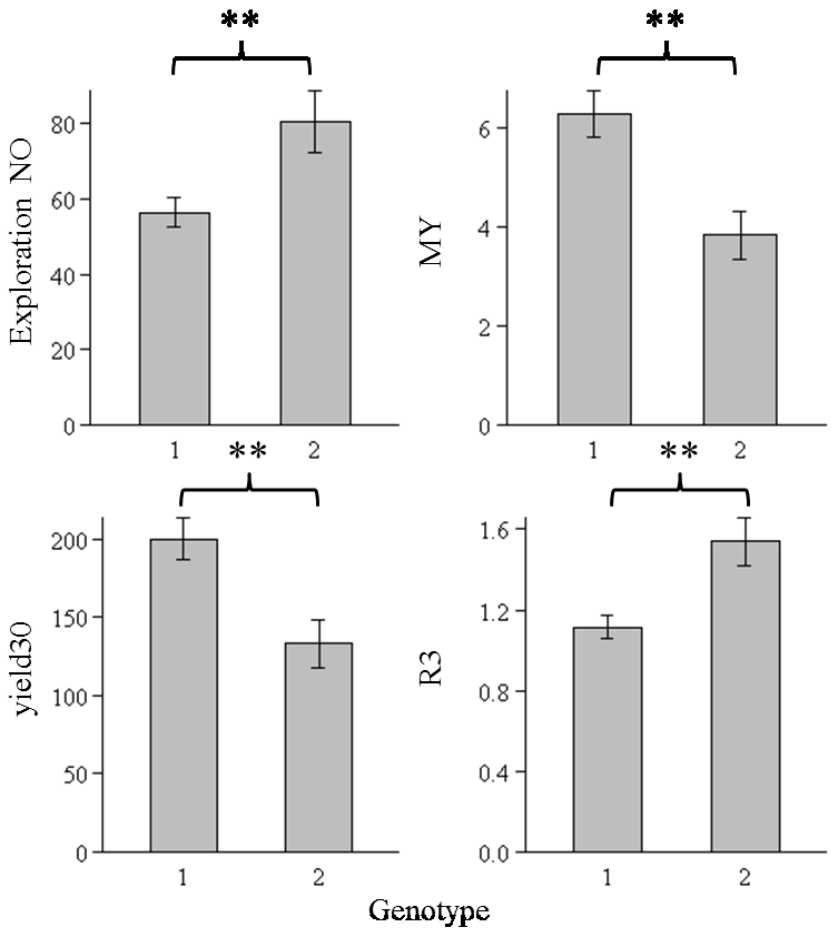

Figure 6.1 Boxplots for Homozygote (1) and heterozygote (2) genotype effects of the three significant SNPs on BTA29 on duration of exploration behaviour in novel-object test (exploration NO), the average daily milk yield (MY), milk yield day 6 to 30 (yield30) and ratio for milk yield three days before and after rehousing (R3). ** represent significant differences with for *P*-value = 0.005

Within this region of LD, three genes *LOC524642* (glycerophosphodiester phosphodiesterase domain containing 4-like), *LOC100849541* (glycerophosphodiester phosphodiesterase domain containing protein 4-like, pseudogene) and *LOC782090* (eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa pseudogene) were localized. In a transcriptome study, Bionaz *et al.* (2012) reported that members of transmembrane protein family glycerophosphodiester phosphodiesterase have functions in bovine lactation. However, in this thesis the transcript of *LOC524642* was not detected as "expressed" in the adrenal cortex hindering a subsequent analysis of this gene at the transcription level.

Considering the relationship between the adrenal cortex and milk performance, Akers *et al*. (1981) suggested that glucocorticoids have a function in lactation initiation. Accordingly in this thesis, most significant correlations were identified for milk performance at the beginning of lactation (yield5) in comparison to the correlation with the other milk performance traits. These 228 identified genes were involved in *eIF2* signalling and protein synthesis. The signalling of peroxisome proliferator-activated receptor (*PPAR*) was significantly enriched by the genes which gene expression was correlated to average milk yield (MY) in the second lactation. Bionaz *et al.* (2013) proposed *PPAR*s as potential targets for fine-tuning metabolism during growth and lactation because they have functions in lipid metabolism, anti-inflammatory response and milk fat synthesis in lactating ruminants. This additional analysis of adrenocortical gene expression and milk performance traits provides evidence for a relationship between adrenal cortex gene expression and milk performance, but a direct link between milk performance and cattle behaviour could not be drawn by the enrichment of pathways involved in glucocorticoid biosynthesis, as assumed.

To sum up, if selection is applied on a particular phenotype, putative effects on other traits are also of interest. Previous results regarding the association between behavioural characteristics and production traits in cattle indicate that the selection of calmer temperaments would be beneficial for productivity and animal welfare. In Chapter 3, the duration of exploratory behaviour was negatively associated with milk performance traits and the results of relationship between temperament types and milk performance indicate that fearful animals (TT1) have lower milk yields compared to the non-fearful cows (TT2 to TT5). This observation is in line with the assumption that stress has a

negative effect on oxytocin release and thus, on milk production (van Reenen *et al.* 2013). The results of this thesis indicate rather no or positive side effects by the selection for cattle temperament on milk performance. Not all SNPs associated with behavioural traits were also significantly associated with milk performance. Further, genotype effects of SNPs that were also significantly associated to milk performance showed a positive relationship between inactivity and milk performance traits. And finally, "fearful/neophobic-alert" cows, which were not desired for selection, had lower milk yields.

Summary

Breeding efforts for cattle behaviour are becoming more relevant due to the effect of behavioural characteristics on production traits and animal welfare, but knowledge about underlying genetic and biological mechanisms is still limited.

In this thesis, different experiments were carried out to give new insights into the biological background of cattle behaviour and its relationship to milk performance in a F2 cross breed cows (German Holstein x Charolais). First, a genome-wide association study was conducted to identify genomic regions with impact on cattle behavioural traits assessed in early life at the age of 90 days and to test for their associations to milk production traits (Chapter 3). In total, 41 single nucleotide polymorphisms (SNPs) distributed over 21 chromosomes were identified to be significantly associated with active, inactive and exploratory behaviour in open-field and novel-object tests. The genetic regions affecting behavioural traits in cattle were specific for the recorded trait and test situation. Of the 9 SNPs which are simultaneously significant for behaviours and milk production, all showed competitive genotype effects for exploratory behaviour and milk production. BTA29, where QTL for cattle temperament and milk yield have been identified before, emerged to be an interesting genomic target region for the joint analysis of cattle behaviour and milk performance.

Furthermore, gene expression profiles of the bovine adrenal cortex, an important tissue in the physiological stress response, of cows slaughtered in the second lactation were analysed between cows classified in five different temperament types in a novel-human test (Chapter 4). 2,944 adrenocortical transcripts were identified to be significantly differentially expressed between temperament types. Especially “fearful/neophobic-alert” animals could be clearly discriminated from others by expression profiles. Significantly altered transcripts between the different temperament types enriched functional processes that are involved in cellular maintenance, proliferation and survival as well as pathways of stress response, for example, “NRF2-mediated Oxidative Stress” and thus highlighted the importance of adrenal cortex development and individual stress reactivity in the context of cattle temperament.

Additional analyses based on the data could reveal that individual differences in temperament have an impact on milk performance, because cows that were more fearful had lower milk yields in comparison to the others. Moreover, these analyses provide evidence that adrenocortical gene expression is correlated to milk performance, especially to milk yield in the first days of lactation.

Zusammenfassung

Die züchterische Modifizierung von Temperament beim Rind gewinnt an Bedeutung da zum einen ein Einfluss auf wichtige Produktionsmerkmale und zum anderen auf das Tierwohl festgestellt werden konnte. Genaue Kenntnisse über den Verhaltensvariationen zugrundeliegende genetische und biologische Mechanismen sind jedoch fehlen jedoch weitestgehend.

In dieser Studie wurden verschiedene Experimente durchgeführt um neue Einblicke in den biologischen Hintergrund von Rinderverhalten und den Zusammenhang mit der Milchleistung in Kühen einer F2 Kreuzung (Deutsche Holstein x Charolais) geben zu können. Zuerst wurde eine genomweite Assoziationsstudie erstellt um genomische Regionen mit einen Einfluss auf Verhaltensmerkmale, die im Kalbesalter von 90 Tagen erfasst wurden, zu identifizieren und deren Einfluss auf Milchmerkmale zu analysieren (Kapitel 3). Insgesamt konnten 41 Einzelnukleotid-Polymorphismen (SNP, engl. single nucleotide polymorphism) verteilt über 21 Chromosomen identifiziert werden, die significant mit aktiven, inaktiven und explorativen Verhalten im ‘open-field’ und im ‘novel-object’ Test assoziiert waren. Die identifizierten genetischen Regionen mit Einfluss auf Verhalten waren spezifisch für die jeweiligen Verhaltensweisen und Testsituationen. Gleichfalls waren 9 SNPs auch nominal signifikant für Milchleistungsmerkmale, die entgegengesetzte Genotypeneffekte für aufgeregtes Verhalten und Milchleistung zeigten. Das bovine Chromosom 29 wurde schon in vorherigen Studien als interessante genomische Region für Rinderverhalten und Milchleistung beschrieben und auch in dieser Analyse zeigten sich signifikante SNPs für Verhalten und Milchleistung.

Weiterhin wurden Genexpressionsprofile der Nebennierenrinde, einem wichtigen Organ der physiologischen Stressantwort, zur Zeit der 2. Laktation, angefertigt und zwischen Kühen die in einem ‚novel-human‘ Test fünf verschiedenen Temperamentstypen zugeordnet wurden, verglichen (Kapitel 4). Dabei wurden 2,944 Transkripte identifiziert die signifikant unterschiedlich zwischen den Temperamentstypen exprimiert waren. Besonders die als „fearful/neophobic-alert“ klassifizierten Kühe unterschieden sich deutlich hinsichtlich ihrer Expressionsprofile von den anderen Tieren. Signifikant

unterschiedliche Transkripte zwischen den Temperamentstypen waren vor allem an funktionellen Prozessen der zellulären Erhaltung, der Proliferation und des Überlebens beteiligt, sowie an Stoffwechselwegen der Stressantwort wie “NRF2-mediated Oxidative Stress”. Dies verdeutlicht den Einfluss der Gewebeentwicklung der Nebennierenrinde und der individuellen Stressanfälligkeit in Hinblick auf Temperament beim Rind.

Zusätzliche Analysen auf Grundlage der vorliegenden Daten konnten zeigen, dass tierindividuelle Temperamentsunterschiede einen Einfluss auf die Milchleistung haben, da ängstlichere Kühe im Durchschnitt weniger Milch gaben, als andere. Zusätzlich konnten Hinweise auf die Bedeutung der Genexpression in der Nebennierenrinde für die Milchleistung insbesondere am Anfang der Laktation gegeben werden.

Appendix

Table S4.1 Loadings of PC1 and PC2 of PCA for NH test

Behaviour trait	**PC1**	**PC2**
Contact-Duration	0.94705	0.05830
Contact-Frequency	0.94720	0.09241
Contact-Latency	-0.94260	-0.13646
Inactivity-Duration	-0.22171	-0.89677
Exploration-Duration	0.07890	0.80112
Exploration-Latency	-0.00272	-0.72105
Grooming-Duration	0.16334	0.39835
Activity-Duration	0.34037	0.79040
Activity-Latency	-0.34251	-0.31350
Running-Duration	0.14909	0.42287
Vocalisation-Frequency	-0.01926	0.33588
Change of segment-Frequency	0.47524	0.75050
Object segment-Latency	-0.88095	-0.24372
Object segment-Duration	0.91188	0.15996
Object neighbouring segment-Latency	-0.71163	-0.32637

Table S4.2 Significantly enriched canonical pathways for the comparison between TT1 and TT2

Ingenuity Canonical Pathways	**-log(*P*-value)**	**Ratio**
Aldosterone Signaling in Epithelial Cells	3.76	0.04
IGF-1 Signaling	3.71	0.05
NRF2-mediated Oxidative Stress Response	3.48	0.04
Myc Mediated Apoptosis Signaling	3.46	0.07
ERK/MAPK Signaling	3.35	0.04
Neurotrophin/TRK Signaling	3.35	0.06
GDNF Family Ligand-Receptor Interactions	3.22	0.06
Insulin Receptor Signaling	3.21	0.04
Melanocyte Development and Pigmentation Signaling	2.95	0.05
VEGF Signaling	2.82	0.05
FcγRIIB Signaling in B Lymphocytes	2.77	0.07
CDK5 Signaling	2.76	0.04
HIF1α Signaling	2.71	0.04
NF-κB Signaling	2.69	0.03
CNTF Signaling	2.63	0.07
Regulation of the Epithelial-Mesenchymal Transition Pathway	2.57	0.03
Synaptic Long Term Potentiation	2.52	0.04
Renin-Angiotensin Signaling	2.52	0.04
IL-2 Signaling	2.50	0.06
CCR3 Signaling in Eosinophils	2.50	0.04
14-3-3-mediated Signaling	2.47	0.04
ErbB2-ErbB3 Signaling	2.45	0.06
ErbB4 Signaling	2.45	0.06
Thrombopoietin Signaling	2.43	0.06
HMGB1 Signaling	2.41	0.04
P2Y Purigenic Receptor Signaling Pathway	2.40	0.04
p70S6K Signaling	2.39	0.04
PI3K/AKT Signaling	2.36	0.03
Cardiac β-adrenergic Signaling	2.33	0.03
Antiproliferative Role of Somatostatin Receptor 2	2.32	0.05
ERK5 Signaling	2.32	0.05
Role of JAK1 and JAK3 in γc Cytokine Signaling	2.32	0.05
GM-CSF Signaling	2.28	0.05
Chemokine Signaling	2.26	0.05
IL-15 Signaling	2.22	0.05
Angiopoietin Signaling	2.21	0.05
IL-4 Signaling	2.21	0.05
Macropinocytosis Signaling	2.21	0.05
Erythropoietin Signaling	2.19	0.05
IL-3 Signaling	2.17	0.04
IL-17 Signaling	2.17	0.04
FLT3 Signaling in Hematopoietic Progenitor Cells	2.15	0.04

JAK/Stat Signaling	2.15	0.04
Prolactin Signaling	2.13	0.04
PEDF Signaling	2.13	0.04
BMP signaling pathway	2.13	0.04
NF-κB Activation by Viruses	2.12	0.04
VEGF Family Ligand-Receptor Interactions	2.10	0.04
LPS-stimulated MAPK Signaling	2.08	0.04
Gap Junction Signaling	2.07	0.03
Ceramide Signaling	2.05	0.04
PDGF Signaling	2.05	0.04
Neuregulin Signaling	1.94	0.04
α-Adrenergic Signaling	1.94	0.04
Spermine Biosynthesis	1.94	0.50
α-tocopherol Degradation	1.94	0.50
Spermidine Biosynthesis I	1.94	0.50
Glycine Degradation (Creatine Biosynthesis)	1.94	0.50
Acute Phase Response Signaling	1.93	0.03
Virus Entry via Endocytic Pathways	1.93	0.04
FAK Signaling	1.93	0.04
ErbB Signaling	1.93	0.04
G-Protein Coupled Receptor Signaling	1.93	0.02
CREB Signaling in Neurons	1.90	0.03
PAK Signaling	1.90	0.04
UVA-Induced MAPK Signaling	1.90	0.04
SAPK/JNK Signaling	1.90	0.04
G Beta Gamma Signaling	1.89	0.04
Natural Killer Cell Signaling	1.85	0.03
Mouse Embryonic Stem Cell Pluripotency	1.85	0.03
EIF2 Signaling	1.85	0.02
Glucocorticoid Receptor Signaling	1.84	0.02
Oncostatin M Signaling	1.84	0.06
Telomerase Signaling	1.83	0.03
T Cell Receptor Signaling	1.81	0.03
Trans, trans-farnesyl Diphosphate Biosynthesis	1.76	0.33
Paxillin Signaling	1.75	0.03
mTOR Signaling	1.74	0.02
Rac Signaling	1.71	0.03
Thrombin Signaling	1.70	0.02
fMLP Signaling in Neutrophils	1.70	0.03
HGF Signaling	1.70	0.03
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	1.69	0.03
NGF Signaling	1.69	0.03
Integrin Signaling	1.69	0.02
Fc Epsilon RI Signaling	1.68	0.03
PKCθ Signaling in T Lymphocytes	1.68	0.03

Role of Tissue Factor in Cancer	1.65	0.03
Geranylgeranyldiphosphate Biosynthesis	1.64	0.25
Actin Cytoskeleton Signaling	1.63	0.02
UVC-Induced MAPK Signaling	1.63	0.05
Gαi Signaling	1.59	0.03
Gα12/13 Signaling	1.58	0.03
IL-6 Signaling	1.58	0.03
PTEN Signaling	1.57	0.03
GNRH Signaling	1.49	0.03
Unfolded protein response	1.47	0.04
Relaxin Signaling	1.47	0.02
UDP-N-acetyl-D-glucosamine Biosynthesis II	1.47	0.17
Actin Nucleation by ARP-WASP Complex	1.44	0.04
Regulation of Cellular Mechanics by Calpain Protease	1.43	0.04
Inositol Pyrophosphates Biosynthesis	1.40	0.14
Regulation of eIF4 and p70S6K Signaling	1.37	0.02
Protein Kinase A Signaling	1.35	0.01
Protein Ubiquitination Pathway	1.34	0.02

Table S4.3 Significantly enriched canonical pathways for the comparison between TT1 and TT3

Ingenuity Canonical Pathways	**-log(*P*-value)**	**Ratio**
Complement System	3.56	0.10
T Helper Cell Differentiation	2.76	0.05
Crosstalk between Dendritic Cells and Natural Killer Cells	2.67	0.05
TR/RXR Activation	2.35	0.04
LPS/IL-1 Mediated Inhibition of RXR Function	2.10	0.02
LXR/RXR Activation	1.99	0.03
Heme Degradation	1.77	0.25
Dendritic Cell Maturation	1.69	0.02
Communication between Innate and Adaptive Immune Cells	1.68	0.04
Hypoxia Signaling in the Cardiovascular System	1.59	0.03
IL-10 Signaling	1.52	0.03
Macropinocytosis Signaling	1.50	0.03
NAD Phosphorylation and Dephosphorylation	1.42	0.11

Table S4.4 Significantly enriched canonical pathways for the comparison between TT1 and TT4

Ingenuity Canonical Pathways	**-log(*P*-value)**	**Ratio**
Protein Ubiquitination Pathway	3.64	0.07
Diphthamide Biosynthesis	2.75	0.67
Assembly of RNA Polymerase II Complex	2.28	0.11
Telomere Extension by Telomerase	2.27	0.20
Nucleotide Excision Repair Pathway	2.08	0.12
Glucocorticoid Receptor Signaling	2.05	0.05
autophagy	1.94	0.11
NRF2-mediated Oxidative Stress Response	1.74	0.06
Regulation of eIF4 and p70S6K Signaling	1.73	0.06
UDP-N-acetyl-D-galactosamine Biosynthesis II	1.71	0.22
CDK5 Signaling	1.65	0.07
PI3K/AKT Signaling	1.63	0.06
Cell Cycle Control of Chromosomal Replication	1.60	0.12
HIF1α Signaling	1.59	0.07
Interferon Signaling	1.51	0.11
TWEAK Signaling	1.40	0.10
Androgen Signaling	1.35	0.06
Flavin Biosynthesis IV (Mammalian)	1.31	0.50
ATM Signaling	1.31	0.07
Neuregulin Signaling	1.31	0.06

Table S4.5 Significantly enriched canonical pathways for the comparison between TT1 and TT5

Ingenuity Canonical Pathways	**-log(*P*-value)**	**Ratio**
Protein Ubiquitination Pathway	7.37	0.09
NRF2-mediated Oxidative Stress Response	2.86	0.06
Aldosterone Signaling in Epithelial Cells	2.66	0.06
CDK5 Signaling	2.07	0.07
UDP-N-acetyl-D-galactosamine Biosynthesis II	1.89	0.22
Cell Cycle Control of Chromosomal Replication	1.85	0.12
Actin Nucleation by ARP-WASP Complex	1.71	0.08
Regulation of Cellular Mechanics by Calpain Protease	1.69	0.08
Neuregulin Signaling	1.65	0.06
PI3K/AKT Signaling	1.57	0.05
Nucleotide Excision Repair Pathway	1.57	0.09
Hypoxia Signaling in the Cardiovascular System	1.56	0.07
DNA Double-Strand Break Repair by Non-Homologous End Joining	1.52	0.14
Pyridoxal 5'-phosphate Salvage Pathway	1.51	0.07
Telomerase Signaling	1.50	0.06
Chemokine Signaling	1.47	0.06
Telomere Extension by Telomerase	1.46	0.13
HIF1α Signaling	1.45	0.05
PPARα/RXRα Activation	1.43	0.04
Angiopoietin Signaling	1.40	0.06
GDNF Family Ligand-Receptor Interactions	1.36	0.06
Cdc42 Signaling	1.32	0.05

Table S5.1 List of annotated transcripts significantly correlated with MY, yield5 and yield30

Transcript ID	r_s	P-value*	Chr	Gene
		MY		
12857098	-0.50	1.83E-04	5	*PPARA*
12793325	-0.48	4.24E-04	21	*FBLN5*
12889267	0.48	4.24E-04	8	*HSDL2*
12889041	0.47	5.91E-04	8	*PLIN2*
12853639	0.44	2.51E-03	4	*PDK4*
12886046	-0.43	4.62E-03	7	*PDGFRB*
12848404	0.42	6.59E-03	4	*HILPDA*
12887525	0.41	1.09E-02	7	*SLC22A5*
12773372	-0.40	1.16E-02	19	*MTMR4*
12746112	-0.40	1.36E-02	17	*PXMP2*
12774536	0.40	1.36E-02	2	*HSPE1*
12859375	-0.39	2.01E-02	5	*A2M*
12749627	0.39	2.13E-02	18	*FCGRT*
12880946	-0.38	2.24E-02	7	*ARHGAP26*
12853157	-0.38	2.40E-02	4	*AEBP1*
12867984	-0.38	2.40E-02	5	*TENC1*
12708125	0.38	2.40E-02	11	*KLF11*
12771254	-0.37	2.96E-02	19	*SLC25A35*
12876146	-0.37	3.07E-02	7	*PCDHGC3*
12792063	-0.37	3.25E-02	21	*SLC25A29*
12891069	-0.37	3.45E-02	8	*DPYSL2*
12732659	-0.36	3.96E-02	15	*OLFML1*
12795827	0.36	4.21E-02	22	*SLC25A20*
12698788	0.36	4.23E-02	11	*LAPTM4A*
12870149	-0.36	4.26E-02	6	*PDGFRA*
12726638	-0.36	4.58E-02	15	*SERPING1*
12684420	-0.36	4.58E-02	1	*HEG1*
12833215	0.36	4.58E-02	29	*CPT1A*
12745302	-0.35	4.82E-02	17	*GUCY1A3*
		yield 5		
12793325	-0.43	1.34E-02	21	*FBLN5*
12857098	-0.43	1.34E-02	5	*PPARA*
12774536	0.42	1.52E-02	2	*HSPE1*
12886046	-0.41	1.52E-02	7	*PDGFRB*
12815354	-0.40	1.56E-02	25	*ARHGAP17*
12764333	-0.39	1.56E-02	19	*CHD3*
12889267	0.39	1.56E-02	8	*HSDL2*
12708452	0.39	1.56E-02	11	*LOC781337*
12773372	-0.40	1.56E-02	19	*MTMR4*
12905415	-0.38	1.64E-02	X	*BT.30403*
12872592	-0.38	1.64E-02	6	*TBC1D14*

12835544	0.38	1.72E-02	3	*RPS27L*
12698788	0.37	2.18E-02	11	*LAPTM4A*
12757631	-0.37	2.18E-02	18	*ZNF792*
12859375	-0.37	2.21E-02	5	*A2M*
12853157	-0.37	2.21E-02	4	*AEBP1*
12867915	0.37	2.21E-02	5	*ALG10*
12684420	-0.37	2.21E-02	1	*HEG1*
12693205	0.37	2.21E-02	10	*RHEB*
12844537	0.37	2.21E-02	3	*RPF1*
12694630	0.37	2.21E-02	10	*SRP14*
12867984	-0.37	2.21E-02	5	*TENC1*
12713095	0.36	2.48E-02	12	*LOC783832*
12872980	-0.36	2.48E-02	6	*MIR2450A*
12785308	0.36	2.48E-02	20	*NADKD1*
12876146	-0.36	2.48E-02	7	*PCDHGC3*
12907714	0.36	2.48E-02	X	*VBP1*
12792315	-0.36	2.48E-02	21	*PPP1R13B*
12730377	0.36	2.60E-02	15	*LOC785951*
12872978	-0.36	2.60E-02	6	*MIR2450B*
12715450	-0.36	2.68E-02	13	*DIDO1*
12686768	0.36	2.68E-02	1	*HMGN1*
12888180	0.36	2.68E-02	7	*LOC782989*
12743889	-0.36	2.72E-02	17	*NOS1*
12895924	0.35	2.74E-02	8	*LOC783838*
12837032	0.35	2.87E-02	3	*ALG14*
12760427	0.35	2.87E-02	19	*LOC100335836*
12693887	0.35	2.87E-02	10	*MED6*
12889041	0.35	2.87E-02	8	*PLIN2*
12854794	0.35	2.87E-02	4	*RHEB*
12856031	0.35	2.87E-02	4	*SHFM1*
12859272	0.35	3.03E-02	5	*BT.7193*
12807525	0.35	3.03E-02	24	*NDUFV2*
12748242	0.35	3.04E-02	17	*ETFDH*
12791383	0.35	3.27E-02	21	*DYNLL1*
12753490	0.34	3.37E-02	18	*ZNF45*
12792590	-0.34	3.38E-02	21	*PEX11A*
12858142	-0.34	3.43E-02	5	*WNK1*
12910529	0.34	3.53E-02	X	*LOC782505*
12691879	0.34	3.57E-02	10	*DAD1*
12838632	0.34	3.57E-02	3	*LAMTOR5*
12863669	0.34	3.63E-02	5	*LOC100335214*
12901276	0.34	3.71E-02	9	*RWDD1*
12832151	-0.33	3.88E-02	29	*APLP2*
12880946	-0.33	3.88E-02	7	*ARHGAP26*
12876682	-0.33	3.88E-02	7	*ARHGAP26*
12785159	0.33	3.88E-02	20	*ATP6V0E1*

12821307	0.33	3.88E-02	26	*C10ORF46*
12825884	-0.34	3.88E-02	28	*HK1*
12733998	-0.33	3.88E-02	15	*LOC100851309*
12733831	0.33	3.88E-02	15	*LOC781022*
12697639	0.33	3.88E-02	10	*LOC784260*
12836672	0.33	3.88E-02	3	*MAGOH*
12870149	-0.33	3.88E-02	6	*PDGFRA*
12789487	-0.33	3.88E-02	21	*RCOR1*
12683135	0.34	3.88E-02	1	*RPL10*
12691842	0.33	3.88E-02	10	*RPS27L*
12744415	-0.33	3.88E-02	17	*SH2B3*
12797726	0.34	3.88E-02	22	*SHFM1*
12747703	-0.33	3.88E-02	17	*TTC28*
12715842	-0.33	3.88E-02	13	*TTPAL*
12800363	-0.33	3.88E-02	22	*VGLL4*
12813124	-0.34	3.88E-02	25	*ZNF394*
12874139	-0.33	3.94E-02	6	*LETM1*
12829389	-0.33	4.02E-02	29	*IGF2*
12716382	-0.33	4.07E-02	13	*DIP2C*
12692267	0.33	4.07E-02	10	*MRPS17*
12808788	-0.33	4.07E-02	24	*SETBP1*
12682582	0.33	4.07E-02	1	*UBL5*
12724888	-0.33	4.07E-02	14	*ZNF696*
12713392	0.32	4.13E-02	13	*LOC100849112*
12742000	0.32	4.16E-02	17	*COX6A1*
12881706	-0.32	4.16E-02	7	*ILF3*
12688847	-0.32	4.16E-02	10	*IVD*
12847740	0.32	4.16E-02	3	*LOC618220*
12709527	0.32	4.16E-02	12	*NDFIP2*
12785489	0.32	4.16E-02	20	*PAIP1*
12872474	-0.32	4.16E-02	6	*PCGF3*
12808812	0.32	4.16E-02	24	*RPL6*
12726638	-0.32	4.16E-02	15	*SERPING1*
12760418	0.32	4.16E-02	19	*USMG5*
12834331	-0.32	4.22E-02	29	*AHNAK*
12741692	0.32	4.22E-02	17	*COX7B*
12702649	-0.32	4.22E-02	11	*ENG*
12703748	-0.32	4.22E-02	11	*ENG*
12749627	0.32	4.22E-02	18	*FCGRT*
12816165	-0.32	4.22E-02	25	*GTF3C1*
12844440	-0.32	4.22E-02	3	*KDM4A*
12754372	-0.32	4.22E-02	18	*KLHL36*
12725711	0.32	4.22E-02	14	*LACTB2*
12737958	0.32	4.22E-02	16	*LOC100295775*
12741812	0.32	4.22E-02	17	*LOC100849169*
12697085	0.32	4.22E-02	10	*LOC614366*

12692478	0.32	4.22E-02	10	*LOC784931*
12708036	0.32	4.22E-02	11	*MEMO1*
12811186	-0.32	4.22E-02	25	*MIR2383*
12730659	-0.32	4.22E-02	15	*PARVA*
12794032	0.32	4.22E-02	21	*RPS17*
12888238	0.32	4.22E-02	7	*TRIM52*
12715184	-0.32	4.22E-02	13	*TSHZ2*
12801023	-0.32	4.22E-02	23	*TXNDC5*
12693896	-0.32	4.27E-02	10	*FOXN3*
12795816	0.32	4.28E-02	22	*ARF4*
12742583	-0.32	4.28E-02	17	*DTX1*
12694003	0.32	4.28E-02	10	*EID1*
12733621	0.32	4.28E-02	15	*IMMP1L*
12866619	-0.32	4.28E-02	5	*TCF20*
12909305	-0.32	4.29E-02	X	*EFNB1*
12721206	0.32	4.29E-02	13	*LOC782266*
12733826	0.31	4.30E-02	15	*LOC782668*
12891069	-0.31	4.33E-02	8	*DPYSL2*
12872964	0.31	4.44E-02	6	*LOC100852331*
12738054	0.31	4.44E-02	16	*USMG5*
12857478	0.31	4.45E-02	5	*CD63*
12882986	-0.31	4.45E-02	7	*NFIC*
12909194	0.31	4.48E-02	X	*MBTPS2*
12773273	-0.31	4.48E-02	19	*SMCR8*
12910319	-0.31	4.51E-02	X	*LOC617499*
12772225	-0.31	4.54E-02	19	*PIP4K2B*
12717341	0.31	4.55E-02	13	*BT.105281*
12754050	0.31	4.55E-02	18	*LOC617986*
12725423	-0.31	4.67E-02	14	*ADCK5*
12771502	-0.31	4.67E-02	19	*FOXK2*
12697838	0.31	4.67E-02	10	*LOC788060*
12843873	0.31	4.67E-02	3	*SCP2*
12784672	-0.31	4.67E-02	2	*TCEB3*
12892597	0.31	4.67E-02	8	*TXN*
12807870	-0.31	4.67E-02	24	*LDLRAD4*
12701582	-0.31	4.69E-02	11	*INPP4A*
12784709	0.31	4.69E-02	2	*SNRPD1*
12855038	-0.31	4.79E-02	4	*ATXN7L1*
12767655	0.31	4.79E-02	19	*GABARAP*
12809653	0.31	4.79E-02	24	*RPL17*
12903951	0.31	4.86E-02	X	*APOO*
12853639	0.31	4.96E-02	4	*PDK4*
12875625	0.31	4.96E-02	6	*SMIM14*
12814951	0.31	4.99E-02	25	*LOC100297793*
		yield30		
12857098	-0.53	4.16E-03	5	*PPARA*

12889041	0.50	1.05E-02	8	*PLIN2*
12886046	-0.48	1.44E-02	7	*PDGFRB*

* FDR-adjusted *P*-value

Table S5.2 Enriched biological functions for adrenocortical transcripts significantly correlated to MY with FDR-adjusted *P*-value < 0.05

Category	**P-value**	**Molecules**
Small Molecule Biochemistry	1.86E-04-3.88E-02	*PPARA, AEBP1, CPT1A, PLIN2, HSPE1, SLC22A5, A2M,P DK4, PDGFRB*
Energy Production	9.53E-04-2.38E-02	*PPARA, CPT1A, HSPE1, SLC22A5, PDK4*
Lipid Metabolism	9.53E-04-3.88E-02	*PPARA, AEBP1, CPT1A, PLIN2, SLC22A5, A2M, PDGFRB, PDK4*
Cell Death and Survival	1.97E-03-3.52E-02	*PPARA, PDGFRA, PCDHGC3, A2M, PDK4, PDGFRB*
Cellular Movement	1.97E-03-3.88E-02	*DPYSL2, PPARA, SERPING1, TNS2, GUCY1A3, FBLN5, PDGFRA, A2M, PDGFRB*
Molecular Transport	2.1E-03-3.88E-02	*DPYSL2, PPARA, AEBP1, CPT1A, FBLN5, SLC25A29, PLIN2, SLC22A5, A2M, PDGFRB, PDK4*
Cell Morphology	3.67E-03-3.88E-02	*PPARA, DPYSL2, AEBP1, PLIN2, A2M, PDK4, PDGFRB*
Cell Cycle	3.67E-03-2.38E-02	*PPARA, PDGFRA, PDGFRB*
Cellular Development	4.53E-03-3.88E-02	*PPARA, DPYSL2, AEBP1, FBLN5, PDGFRA, A2M, PDK4, PDGFRB*
Cellular Growth and Proliferation	4.53E-03-3.67E-02	*DPYSL2, PPARA, FBLN5, PDGFRA, A2M, PDK4, PDGFRB*
Cell Signaling	5.19E-03-5.19E-03	*PDGFRA, PDGFRB*
Cell-To-Cell Signaling and Interaction	7.37E-03-3.34E-02	*PPARA, GUCY1A3, HEG1, PDGFRA, PCDHGC3, A2M, PDGFRB*
Carbohydrate Metabolism	8.45E-03-3.52E-02	*PPARA, CPT1A, PLIN2, PDK4*
Cellular Assembly and Organization	8.45E-03-3.88E-02	*PPARA, DPYSL2, PLIN2, HEG1, PCDHGC3, A2M, PDGFRB*
Cellular Compromise	8.45E-03-3.88E-02	*PPARA, DPYSL2*
Cellular Function and Maintenance	8.45E-03-3.34E-02	*DPYSL2, HEG1, PCDHGC3, A2M, PDGFRB*
Nucleic Acid Metabolism	8.45E-03-3.67E-02	*PPARA, CPT1A, HSPE1, SLC22A5, PDK4*
DNA Replication, Recombination, and Repair	1.08E-02-3.03E-02	*FBLN5, GUCY1A3, PDGFRA, A2M, PDGFRB*
Vitamin and Mineral Metabolism	1.37E-02-2.38E-02	*PPARA, PLIN2*
Post-Translational Modification	1.81E-02-1.81E-02	*PLIN2*
Gene Expression	2.15E-02-3.03E-02	*PPARA, PDGFRB*

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