Genetic Analysis of Feather Pecking Behavior in Laying Hens

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Abstract. This thesis describes the genetic analysis of feather pecking behavior in laying hens. Feather pecking (FP) is a major welfare problem in laying hens. In the European Union, legislation concerning animal housing is becoming more strict, because of increasing concern for animal welfare. Conventional cage systems will be banned in 2012 and there is a clear movement towards alternative housing systems such as modified cages and large group housing systems. Unfortunately, in large group housing systems FP is difficult to control. To study the genetics of FP behavior and open-field behavior, an F_2 population of 630 hens coming from two commercial laying lines was generated. All F_2 animals were tested for their FP behavior in a social FP test as well as for their behavior in an open-field test at both young and adult age. The F_0, F_1 and F_2 generations were genotyped with 180 microsatellite markers. Gentle FP was heritable at both young and adult age whereas severe FP was not. Heritability of open-field behavior at young age was higher than at adult age.

A QTL analysis, using a half-sib model and a line-cross model was performed for different types of pecking behavior such as gentle FP, severe FP, aggressive pecking and toe pecking, as well as for receiving pecks and open-field behavior. For gentle FP suggestive QTL were identified at young age at GGA10 and at adult age at GGA2. In addition, for severe FP at adult age, a significant QTL was detected at GGA2. Parallel to this, using a comparative mapping approach, the glucocorticoid receptor gene was mapped to GGA13. This chromosome was not detected in the QTL study which makes this gene less likely to be a candidate gene for FP behavior.

For receiving gentle FP, a significant QTL was detected on GGA1 at young age and at adult age a suggestive QTL was detected on GGA5. For open-field behavior at young age a significant QTL was detected on GGA4 and at adult age a significant QTL was detected on GGA4 as well. The QTL are more than 100cM apart, therefore, it is not likely that these QTL share the same underlying genes. Interestingly, these results indicate that pecking behavior as well as open-field behavior at young age is regulated by different genes than at adult age. The QTL for severe FP and gentle FP offer the possibility to identify genetic markers for FP behavior, which may be used in genetic improvement programs.

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CHAPTER 1

GENERAL INTRODUCTION

Behavior is a complex phenotype for genetic analysis. There are different animal models to study behavior. The fruitfly (*D. melanogaster*) has been used as a model organism to study developmental and anatomical traits. However, *D. melanogaster* has been used also to study different behaviors like the circadian rhythm (wake-sleep cycle), courtship behavior, learning and memory, and feeding and foraging behavior. For these behaviors different genes have been identified (see for a review: Sokolowski, 2001). In mouse and rat behavioral phenotypes are often guided by analogous behavior observed in human psychiatric patients. Different types of behavior have been studied in mice and rats, like the circadian rhythm, aggression, drug preference, drug response, emotion, learning, and motor activity (Flint, 2003). Several quantitative trait loci (QTL) influencing these behaviors were discovered (see for a review: Flint, 2003). The examples mentioned above indicate that behavior has a genetic component. The fast development of molecular genetics enables studying behavior genetics also in farm animals.

Genetic selection for increased and economical efficient productivity in farm animals has been successful in the past 50 years (see for an example in broilers: Havenstein et al., 1994). Laying hens are selected for feed efficiency, egg production traits - such as number of eggs, egg weight, and egg shell quality - and early sexual maturation. As a result also behavior changes, e.g., large differences exist between laying hens and the Red Junglefowl (Schütz and Jensen, 2001). A White Leghorn laying hybrid shows less foraging behavior and is less involved in social interactions when compared to the Red Junglefowl in captivity (Schütz and Jensen, 2001). This indicates that selection for increased productivity resulted in changes of behavior. Increased incidences of pecking behavior and cannibalism may occur as a correlated trait with the selection for increased egg production and early maturation as well (Bhagwat and Craig, 1977; Craig et al., 1975). Furthermore, differences in open-field behavior (Jones et al., 1995) and feather pecking (FP) (Hughes and Duncan, 1972; Bessei, 1984; Blokhuis and Beutler, 1992) were observed between chicken lines selected for production traits.

Feather pecking behavior

Feather pecking is defined as pecking at and pulling out the feathers of a conspecific. Different forms of pecking exist. Each form may have its own underlying motivation (Savory, 1995). FP occurs both in cage systems and in free-range systems. However, FP in free-range systems is a more important issue, because in these housing systems FP is more difficult to

control. Nevertheless, free-range housing systems become more of interest due to the legislation in the European Union (Council directive 1999/74/EC). FP hampers the welfare of the chickens seriously. FP results in denuded areas and causes pain in the chickens (Gentle and Hunter, 1990). Denuded areas and damaged feathers may trigger more pecking, and ultimately, FP behavior leads to cannibalism. Cannibalism is the stage where birds are pecked to death and partly consumed (Savory, 1995; McAdie and Keeling, 2000). Beside the impaired welfare of the chickens, there is also an economic loss for the farmer due to the higher mortality and feed costs (Blokhuis and Wiepkema, 1998).

An outbreak of FP in a flock is unpredictable. There are, however, several environmental factors which play a role in triggering FP behavior, e.g., a bigger group size, or higher stocking density, or a high light intensity increases FP, whereas availability of litter on the floor reduces FP (Hughes and Duncan, 1972; Blokhuis and Arkes, 1984; Bilčík and Keeling, 1999; Green et al., 2000; Appleby et al., 2002). Dietary composition and food form (pelleted vs mash) influence pecking behavior as well, e.g., supplementation of L-tryptophan reduces FP and with regard to food form pecking behavior is more pronounced when fed on pellets than with mash food (Savory and Mann, 1997; Savory et al., 1999; Aerni et al., 2000; McKeegan et al., 2001). Beside environmental influences on FP, line differences in FP behavior (Hughes and Duncan, 1972) suggest the existence of a genetic component as well. More detailed studies focussing on the genetics of FP behavior showed that the heritability of FP behavior is in the range of 0.05 to 0.50 (Cuthbertson, 1980; Bessei, 1984; Kjaer and Sørensen, 1997; Bessei et al., 1999). In addition, divergent selection on FP behavior is feasible using direct observations (Kjaer et al., 2001). Hens were selected for FP (high pecking line; HP line) and against FP (low pecking line; LP line) based on "number of bouts performed". A bout is the time a bird is continuously pecking at the conspecific. In three generations significant differences were observed in number of bouts, number of pecks as well as in the proportion of hens performing FP between the HP and LP line (Kjaer et al., 2001). Another experiment (Muir, 1996) using group selection, resulted in an rapid decrease in mortality due to 'beak-inflicted injuries' - a combination of all types of pecking including cannibalism - already after one generation of selection. Therefore, Muir (1996) suggested that a major gene was involved in pecking behavior.

A direct comparison of heritability estimates between studies is difficult because different measures are used to quantify FP or FP damage based on plumage condition. The different tests can be: 1) direct observations in the flock (Cuthberson, 1980; Bessei, 1984); 2)

pecking directed at a bunch of feathers in an individual context (Bessei et al., 1999). This test was automated using a peck-o-meter. Chicken show strong interest in feathers presented by the observer. The pecks directed at the bunch are a measure for FP behavior in the homepen; 3) direct observation in a social context, i.e., 20 birds are caught from their homepen and transferred to a test cage (Kjaer and Sørensen, 1997); and 4) direct observations in a social context with a bunch of feathers (Rodenburg and Koene, 2003). When comparing the different tests, it was concluded that 'gentle and severe FP and bunch pecking in the social test corresponded best to FP in the homepen, whereas bunch pecking in the individual context did not' (Rodenburg and Koene, 2003).

Coping styles

Animals can be classified according to their coping style. There are two coping styles defined in rodents: a pro-active style and a re-active style (Koolhaas et al., 1999). Pro-active animals have an active behavioral response (fight or flight) and a low adrenocortical response to a stressor. Re-active animals, on the contrary, have an inactive behavioral response (conservation or withdrawal) and a high adrenocortical response to a stressor (Koolhaas et al., 1999) (Table1).

TABLE 1. Physiological and neuroendocrine differences between pro-active and re-active animals (adapted from Koolhaas et al., 1999).

Physiological and neuroendocrine characteristics		
	Pro-active	Re-active
HPA axis activity	Low	Normal
HPA axis reactivity	Low	High
Sympathetic reactivity	High	Low
Parasympathetic reactivity	Low	High
Testosterone activity	High	Low

Based on the comparison of two commercial laying lines for their pecking behavior, two lines were characterized as a low FP (LFP) line and a high FP (HFP) line (Blokhuis and Beutler, 1992; Rodenburg and Koene, 2003). When applying the theory of coping-styles to these two laying lines, the LFP animals could be characterized as having a re-active coping style (LFP animals have a high corticosterone response to a stressor) and the HFP animals could be characterized as having a pro-active coping style (HFP animals have a low corticosterone response to a stressor) (Korte et al., 1997; van Hierden et al., 2002).

The role of corticosterone in the stress response system displays two distinct modes. First, a pro-active mode, i.e., corticosterone maintains basal activity of the HPA-system and regulates the threshold of the system's response to stress. Second, a re-active mode, i.e., corticosterone facilitates an animal's ability to cope with, adapting to, or recovering from a stressful situation by reducing or terminating stress-induced HPA-axis activation (de Kloet et al., 1998). Corticosterone does not necessarily cause a behavioral change of an animal, but influences the information processing which can result in an appropriate behavioral response. The mineralocorticoid receptors and glucocorticoid receptors regulate behavioral processes in concert. Mineralocorticoid receptors influence effects of corticosterone on appraisal of information and response selection, whereas glucocorticoid receptors influence processes underlying consolidation of acquired information (see for an extensive review: de Kloet et al., 1998). Taking into account the role of corticosterone and the mineralocorticoid receptor/glucocorticoid receptor mediated influence on behavior, it was hypothesized that 'FP behavior represents a failure of normal adaptive mechanisms to operate, and that glucocorticoids play a crucial role in this process' (Korte et al., 1997).

To characterize an animal according to the coping style theory, an open-field test can be used. The open-field test is commonly used as a measure for the supposed emotional state of an animal. The measurement is relatively easy: an animal is placed in an open-field and the activity of the animal is recorded. However, Candland and Nagy (1969) suggested that there is a large difference between rodent and chicken behavior observed in the open-field test. When a rodent shows active behavior in a newly encountered situation, a chicken shows freezing (Candland and Nagy, 1969). Differences observed for open-field behavior between chicken lines suggest a genetic component (Webster and Hurnik, 1989; Jones et al., 1995). To date not many studies have investigated the genetics of open-field behavior in chicken. There is evidence, however, that open-field behavior is heritable (Faure, 1981; Webster and Hurnik, 1989). In addition, divergent selection for open-field behavior in two-day-old chicks was feasible. In eight generations selection, large differences between the two divergent lines were observed for general activity, jumps, vocalizations, and the number of cells crossed in the open-field (Faure, 1981).

Genetics of the chicken

Insight in the genetic mechanism underlying FP behavior and correlation between FP behavior and other behaviors and traits, e.g., open-field behavior as well as stress response,

immune response and production traits, may be of help to describe the phenomenon of FP behavior more precisely and in the end reduce the FP problem in practice. In the last two decades tools to dissect the molecular basis of quantitative traits have been developed in chicken. The chicken genome consist of 38 pairs of autosomes and two sex chromosomes (Z and W). The genome size is approximately 1.2 10^9 bp (Stevens, 1986). The females are heterochromosomal (ZW) and males are homochromosomal (ZZ). The development of genetic markers have facilitated genome mapping. Different types of genetic markers have been developed, e.g., restriction fragment length polymorphisms, microsatellite markers ($(T)_n$, $(CA)_n$ or $(CAC)_n$), amplified fragment length polymorphisms, and single nucleotide polymorphisms (SNP). These genetic markers have been used to build genetic linkage maps. In chicken, three reference families were used as a mapping population: the Compton reference population (Bumstead and Palyga, 1992); the East Lansing reference population (Crittenden et al., 1993); and the Wageningen reference population (Groenen et al., 1998). The mapping results of these three reference populations were combined into a consensus linkage map consisting of, approximately, 1900 markers and is 3800 centiMorgan in length (Groenen et al., 2000).

A linkage map is an essential step in the search for genes involved in economically important traits. Quantitative traits are controlled by a large number of loci (QTL). Mapping of QTL is conceptually simple. One can use two lines - preferably inbred, however, this is not likely to occur in farm animals - differing in the trait of interest. A cross is made to generate the F_1 population. Animals from this F_1 population are used to make a backcross- or an F_2 intercross mapping population. All animals from the mapping population are phenotyped, i.e., characterized for the trait of interest, and genotyped, i.e., characterized for multiple loci throughout the genome. The idea of making an F_2 - used in this thesis - is that the genotypes of the founder lines are mixed due to recombination. As an example for one homozygous locus: line 1 has genotype AA and line 2 has genotype aa. The first cross to generate the F_1 animals results in animals having genotype class: Aa. In a next (inter)cross to generate the F_2 animals, the genotype classes of the F_2 animals can be either aa, Aa, or AA. The animals from the mapping population are divided into genotype classes. It is tested whether there is a significant phenotypic difference between the genotype classes. If there is a difference, than the QTL is associated with the marker. In the study described in this thesis interval mapping is used, which allows to give an estimate for the location of the QTL. Estimation of the QTL position is not possible or at least very inaccurate using single marker analysis (van Arendonk

and Bovenhuis, 2003). The advantage of using a QTL approach is that no a priori assumptions are made concerning candidate genes involved in the trait of interest.

An initial QTL mapping experiment will position the QTL in a chromosomal region of approximately 50 cM. The genetic to physical ratio is on average 396 kb/cM for macrochromosomes and 150 to 250 kb/cM for microchromosomes. The number of genes is on average one gene per 22 kb for the macrochromosomes and one gene per 17 kb for the microchromosomes (Schmid et al., 2000). Once a QTL is detected it is of interest to identify the underlying genes. Therefore, large insert libraries such as a yeast artificial chromosome library (Toye et al., 1997) and a bacterial artificial chromosome library (Crooijmans et al., 2000) were developed to make a physical map of the chicken genome. In addition, a chicken radiation hybrid panel was developed, which is a valuable tool for mapping genes (Morisson et al., 2002). cDNA collections of the chicken are an additional tool to generate chicken specific gene sequences (Abdrakhmanov et al., 2000). These sequences can be of help to improve the chicken gene map, when mapped on the linkage- or the radiation hybrid map. To date, more than 300 genes were mapped on the chicken linkage map (Groenen et al., 2000). More detailed comparative maps using the Wageningen bacterial artificial chromosome library were developed for GGA10 (Crooijmans et al., 2001), GGA15 (Jennen et al., 2003), and GGA24 (Jennen et al., 2002). Although large conserved segments between chicken and human can be identified (Burt, 2002), more detailed gene mapping show multiple inter- and intrachromosomal rearrangements (Crooijmans et al., 2001; Jennen et al., 2002; 2003). Despite the effort of making high density comparative maps between chicken, human and mouse, the gene density is still too low to identify feasible candidate genes for the traits of interest. Recently, sequencing of the chicken genome has started. The draft sequence is expected to be finished by the end of 2003. The chicken sequence is essential for the identification of the candidate gene(s) in the QTL region.

Description of the chicken lines used in this study

Two commercial White Leghorn laying lines provided by Hendrix Poultry Breeders BV, Boxmeer, The Netherlands were used as the basis of this research. Both lines were selected for a combination of egg production traits. The exact breeding goal was not disclosed to the researchers. Blokhuis and colleagues have used these chicken lines in their research and showed that these lines differ in their propensity to FP (Blokhuis and Beutler, 1992). These lines were called the high FP- (HFP) and low FP (LFP) line. The HFP birds grew faster and were one week earlier in laying their first egg compared to the LFP birds (T. B. Rodenburg, personal communication). The LFP line showed a shorter duration of immobility reaction in the tonic immobility test than the HFP line at 14 weeks of age (Blokhuis and Beutler, 1992). In addition, these lines also differed in their reaction to open-field behavior at 7 days of age, however, no line differences were found for the tonic immobility test at approximately 5 weeks of age (Jones et al., 1995). Two years later, Korte et al. (1997) showed that the HFP and LFP lines also differed in their physiological reaction to a stressor (manual restraint test). The HFP line had a lower corticosterone response and a higher noradrenaline response than the LFP line. When starting the research described in this thesis, the line differences were verified. It turned out that there was still a significant difference between the HFP and LFP lines for FP behavior using the social FP test (Rodenburg and Koene, 2003). However, when there was full light and no wood shavings available on a slatted floor, there was no difference in FP behavior between the lines. In that case FP was so severe in both lines that there was no visible difference between the plumage condition of the LFP- or HFP line (Rodenburg et al., 2002). Concerning open-field related response (e.g., vocalizations), LFP animals tended to vocalize more at young age than the HFP birds, but from week 10 this reaction was opposite. The reaction to the tonic immobility test was not consistent between the two lines in repeated tests. With regard to the stress response the HFP birds showed a lower corticosterone response to a stressor than the LFP birds for young as well as adult birds (van Hierden et al., 2002). In the same study, a significant difference between the HFP and LFP line was found for the neurotransmitters dopamine (DA) and serotonin (5-HT) turnover in the brain and their metabolites. Both DA and 5-HT turnover were significantly lower in the HFP line compared to the LFP line.

Based on the verification of the traits described above it was decided to use the social FP test, the open-field test and the manual restraint test in an F_2 mapping population generated from the two commercial white leghorn laying lines.

Aim and outline of this thesis

The aim of the work described in this thesis is to identify genetic factors underlying FP- and open-field behavior in laying hens. To verify whether FP- and open-field behavior were heritable, the heritability was estimated for different forms of FP behavior and open-field behavior (Chapter 2). Identification of genetic markers linked to FP behavior and stress response would be a first step to the identification of underlying genes (Chapter 3). To verify the hypothesis whether the glucocorticoid receptor (NR3C1) was involved in the stress response to manual restraint this gene was mapped on the chicken linkage map on GGA13. This study was extended by making a detailed chicken-human comparative map between GGA13 and HSA5 (Chapter 4). In the search for a viable way to improve animal welfare in poultry, insight in the genetic architecture of receiving FP might be useful in comparing the peckers with the receivers. Therefore, a QTL analysis was performed for being pecked (Chapter 5). Because the HFP and LFP differed in their open-field response behavior as well, the F_2 animals were subjected to an open-field test. Chapter 6 describes the identification of QTL involved in open-field behavior. Finally, in chapter 7 the results of this thesis are discussed.

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CHAPTER 2

Heritability of Feather Pecking and Open-Field Response in Laying Hens at Two Different Ages

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ABSTRACT

The objective of the current study was to estimate heritabilities (h^2) of feather pecking and open-field response in laying hens at two different ages. An F_2 cross, originating from a high and a low feather pecking line of laying hens, was used for the experiment. Each of the 630 birds of the F_2 cross was subjected to an open-field test (individual, 10 min) at 5 and 29 wk of age, and to a social feather pecking test (groups of 5 birds on wood shavings, 30 min) at 6 and 30 wk of age. Both tests were performed in a square open-field (1.25 x 1.25 m). Behavior was recorded directly from a monitor. Heritabilities of feather pecking and open-field behaviors were calculated. In the open-field test at 5 wk of age, high h^2 were found for most traits, ranging from 0.20 for the frequency of flying to 0.49 for number of steps. In the social test at 6 wk, gentle feather pecking (0.12) and ground pecking (0.13) were found to be heritable. When both tests were repeated at 29 and 30 wk of age, h^2 estimates were lower for the open-field test, ranging from 0.10 for duration of sitting to 0.20 for latency to first step. In the social test, however, higher h^2 estimates of 0.15 for gentle feather pecking and 0.30 for ground pecking were found compared with 6 wk of age. In conclusion, gentle feather pecking and open-field behaviors may be used in selection against feather pecking.

(*Key words*: heritability, feather pecking, open-field, laying hens, behavior genetics)

INTRODUCTION

Feather pecking impairs animal welfare and results in higher feeding costs and increased mortality rates in laying hens. It is characterized by pecking at- and pulling of feathers of other birds. Feather removal has been shown to be painful (Gentle and Hunter, 1990) and results in bald patches. This feather damage as a result of feather pecking can lead to a heat loss resulting in 20% higher energy requirements (Blokhuis and Wiepkema, 1998). The bald patches may attract tissue pecking, resulting in wounded birds that may be pecked to death (Savory, 1995). Feather pecking is a problem in all current housing systems for laying hens, but in large group housing systems the problem is more difficult to control. The current development in the European Union from beak-trimmed birds in battery cages towards large group housing with birds with intact beaks asks for tools to control feather pecking. In the United States, guidelines developed by large fast-food chains in cooperation with scientists (Rahn, 2001) may steer future egg production systems in the same direction as in the European Union.

Line differences in both plumage condition (Ambrosen and Petersen, 1997; Wahlstrom et al., 2001) and in feather pecking behavior (Hughes and Duncan, 1972) suggest a genetic background. Direct selection has been shown to be feasible, using either individual selection against feather pecking (Kjaer et al., 2001) or group selection against mortality (Craig and Muir, 1993; Muir, 1996). Craig and Muir (1993) reported a high realized family heritability (h^2) of 0.65 for days without beak-inflicted injuries. Three studies have reported h^2 of feather pecking based on direct observation of pecking behavior (Cuthbertson, 1980; Bessei, 1984; Kjaer and Sørensen, 1997). In these studies, h^2 for performing feather pecking ranged from 0.07 to 0.56 and for receiving feather pecking from 0.00 to 0.15. None of these studies distinguished between the different forms of feather pecking, i.e. gentle and severe feather pecking (Savory, 1995). This distinction may be important, as gentle and severe feather pecking may have different ways of developing and may be differently affected by genetic and environmental factors. Recently, it has been suggested that gentle feather pecking at young age may develop into stereotyped gentle feather pecking or into severe feather pecking in adult laying hens by either increased intensity or increased severity of bird-to-bird pecks (McAdie and Keeling, 2002).

Feather pecking has also been associated with fearfulness (Hughes and Duncan, 1972) and, more recently, with open-field response (Jones et al., 1995) and coping strategy (Korte et al.,

1997). The open-field test has been used for the study of emotional reactivity and motivation in laboratory animals, but also in poultry (Candland and Nagy, 1969). Gallup and Suarez (1980) proposed that open-field behavior in poultry is a compromise between opposing tendencies in a bird to return to its flock-mates and to minimize detection by predators (experimenter). Some studies have estimated h^2 for open-field behaviors, with estimates for overall locomotion ranging from 0.08 to 0.49 and for defecation from 0.06 to 0.10 (Boyer et al., 1970; Faure, 1981; Webster and Hurnik, 1989). Jones et al. (1995) showed that birds from a low feather pecking line vocalized and walked sooner in an open-field than birds from a high feather pecking line, reflecting differences in social motivation to return to their flock-mates. Thus the open-field response may be useful as a predictor of feather pecking behavior and the open-field test may be used to select against feather pecking.

The aim of the present experiment was to estimate h^2 of open-field response at 5 and 29 wk of age and of feather pecking at 6 and 30 wk of age. A distinction was made between gentle and severe feather pecking, as they may be differently affected by genetic and environmental factors. The current study may give a better understanding of the possibilities to select against feather pecking either by direct observation of feather pecking or selection on related characteristics using the open-field test. This may eventually enable the breeding of birds that are better adapted to future housing systems.

MATERIALS AND METHODS

Genetic Stock and Population Structure

Two selection lines from a commercial breeder were used for this experiment. These lines have been selected for production related traits, but also differ consistently in feather pecking behavior: the high and low feather pecking lines (Rodenburg and Koene, 2001; Riedstra and Groothuis, 2002; Van Hierden et al., 2002). A reciprocal cross of these lines was made: six high feather pecking males and six low feather pecking females were used to produce the high x low cross and six low feather pecking males and six high feather pecking females were used to produce the low x high cross. This resulted in an F_1 generation consisting of 120 animals. From this generation, 7 males and 28 females were randomly selected and mated to create an F_2. On average, each female produced 23 female offspring and each male produced 90 female offspring. The total number of female birds in the F_2 generation was 630.

Housing and Management

Birds arrived at the experimental farm as day-old chicks in 5 batches. Every 2 wk one batch of about 125 animals was delivered (Weeks 30, 32, 34, 36 and 38 in the year 2000). Each batch was allocated at random to two floor pens with an average of 63 birds (between 55 and 70) in each pen, ten pens in total. Each pen measured 4.75 x 2 m and was supplied with wood shavings, two heating lamps per pen for warming and ad libitum feed (152 g/kg crude protein, 2.817 kcal/kg metabolizable energy) and water. From 0 to 4 wk of age the birds had continuous light (one red heating lamp and two 40 W light tubes per pen). From 5 through 15 wk of age the heating lamps were removed and the birds had 8 h light between 8:00 and 16:00 h. From 16 wk of age onwards, the light period was extended with 1 h per wk, until birds had 16 h light between 3:00 and 19:00 h at 24 wk of age. The birds were not beak-trimmed. Each bird was individually marked with a wing tag. Males not excluded from the experiment at 1 d of age, due to errors in gender determination, were removed at 5 wk of age. At 18 wk of age, each pen was supplied with laying nests and perches. During the experiment, the mortality rate was 13%, partly because of a coccidiosis infection when the birds were about 20 wk of age. This infection mostly affected Batches 4 and 5, resulting in more space per bird in these groups. All groups were treated with vitamins and after that treatment no problems with coccidiosis were observed thereafter. The Wageningen University Committee on Animal Care and Use has approved this experiment.

Open-Field Test

At the age of 5 and 29 wk all birds were tested individually in the open-field test for 10 min. The open-field consisted of a 1.25 x 1.25 m observation pen, which was divided in 25 squares by white markings (5 x 5), measuring 25 x 25 cm each. The front wall was made of Perspex, through which a camera recorded the area of the pen. The observer could then record the behavior from a video-screen in an adjacent room. General activity and vocalizations were recorded according to the ethogram (Table 1).

TABLE 1. Ethogram of the open-field test.

Behavior	Description
Sitting	Sitting with breast and belly on the floor
Standing	Standing, feet/legs, but not belly on the floor
Walking	Locomotion, minimum of 2 steps
Step	Number of steps
Flying	Flapping wings, no contact with floor
Distress call[1]	Distress call (peep)
Alarm call[2]	Alarm call (kot kot kot kodeeek)
Alarm call note[2]	Alarm call note (kot)
Defecating	Defecating

[1]Only included in the ethogram at 5 wk of age
[2]Only included in the ethogram at 29 wk of age

Latencies, durations, and frequencies of all common behaviors were recorded using focal sampling. The catching procedure consisted of entering the homepen, passing a number of birds, walking back to the door, and capturing the first bird in sight, alternating between the two groups of one batch. To avoid unnecessary stress of the individual bird before the test, it was transported to the observation pen in a box. The bird was placed in the middle of the observation pen. The room with the observation pen was dark until the start of the test. At 5 wk of age, the same person conducted all behavioral observations, but a different experimenter tested and handled Batch 2. At 29 wk of age two different persons performed the open-field observations, after their behavioral recording methods were brought into conformity with each other. Birds were tested between 8:45 and 16:15 h. After the test, each bird was marked with a color across the back (just behind the neck) for identification purposes in the social test. For this purpose 5 different colors (red, green, blue, purple, and orange) were alternated. Earlier observations showed that these colors did not affect feather pecking behavior or aggressive interactions (T.B. Rodenburg, unpublished data).

Social Test

At 6 and 30 wk of age all birds were tested in groups of 5 in the social test for 30 min; this test was described previously in Rodenburg and Koene (2001). The social test was executed in the open-field observation pen with wood shavings on the floor. Five birds with different colors were captured from one pen of a batch, alternating between the two pens. They were identified and transported to the observation pen in a crate, where they were placed in darkness. The test began with switching on the light. After 5 min, a sound signal was produced to avoid birds being inactive for 30 min. Body weight of each bird was

recorded after the test, and birds were marked with a black dot. One person handled and observed all the birds in the social test. The birds were all tested within the time period 8:45 through 15:45 h. Pecking behavior was sampled directly using behavior sampling, i.e. sampling all occurrences of some behaviors (Martin and Bateson, 1993). For feather pecking and aggressive pecking both the actor and the receiver were recorded. The ethogram is described in Table 2.

TABLE 2. Ethogram of the social test.

Behavior	Description
Gentle feather peck	Gentle feather peck, no reaction receiver, and neck still
Gentle bout	Bout of gentle feather pecks
Severe feather peck	Severe feather peck, reaction receiver, neck moves
Severe bout	Bout of severe feather pecks
Aggressive peck	Dominance peck, directed at head, neck, or back
Ground bout	Bout of ground pecks. Pecks directed at ground

Statistical Analyses

Exploratory analyses were performed using SAS® (SAS Institute, 1996) by use of the general linear model procedure to estimate sire and dam variances. For the analysis of the open-field test, sire (7 levels) and dam (28 levels; nested within sire) were included in the model as random effects, whereas pen (10 levels), and time of testing (5 levels at 5 wk and 4 levels at 29 wk) were included as fixed effects. The social test was analyzed with sire (7 levels), and dam (28 levels; nested within sire) included in the model as random effects, and test group (129 levels at 6 wk and 112 levels at 30 wk) included as fixed effect. Body weight was analyzed in a separate model, including the effects of pen (10 levels) and day (3 levels). Variances were estimated based on information of both sire and dam components of variance. Heritability estimates based on the sire component and the dam component respectively, were then calculated as:

$$h^2_{SIRE} = 4\,\sigma^2_{SIRE}/\sigma^2_P \text{ and } h^2_{DAM} = 4\,\sigma^2_{DAM}/\sigma^2_P.$$

Subsequently an analysis was performed using an animal model and the ASREML software package (Gilmour et al., 2000). Univariate analyses were performed on all recorded traits to estimate the phenotypic and additive genetic variance. For this analysis the following mixed model was used:

$$Y = X\beta + Zu + e$$

where Y is a vector of observations, X is the design matrix for fixed effects, ß is the vector of fixed effects, Z is the design matrix for random effects, u are the random effects with var (u) = $A\sigma^2_u$ and e are the residuals with var (e) = $I\sigma^2_e$. The fixed effects for the open-field test were pen with 10 levels and time of testing with 5 levels at 5 wk of age and 4 levels at 29 wk of age. The fixed effect for the social test was test-group with 129 levels at 6 wk of age and 112 levels at 30 wk of age. The fixed effects for both tests were the same as used for the exploratory analysis in SAS® (SAS Institute, 1996).

RESULTS

Description of Traits

Means and standard deviations of indicator traits in the open-field test and in the social test at both ages are presented in Table 3. In the open-field test at 5 wk of age, birds on average spent 430 s sitting (72% of total time). If birds became active, they started to vocalize, than they stood up and walked. On average, they uttered about 56 distress calls and walked 30 steps. Some birds also tried to fly out of the open-field (mean 0.27 flights per bird). At 29 wk of age, birds spent less time sitting (35% of total time) and walking (10 steps) and more time standing (60% vs. 22% of total time) than at 5 wk of age. In the social test, levels of gentle and severe feather pecking and ground pecking were higher at 30 wk than at 6 wk of age. Average body weight increased from 368 g at 6 wk to 1,606 g at 30 wk. Most distributions of behavioral traits were skewed to the right with many observations with value zero.

TABLE 3. Means and standard deviations of indicator traits in the open-field test at 5 wk and 29 wk of age and in the social test at 6 wk and 30 wk of age.

Trait	5 wk		29 wk	
Open-field test	Mean	SD	Mean	SD
Duration of sitting (s)	431	201	209	252
Duration of standing (s)	134	163	357	241
Latency to first call[1] (s)	334	230	467	191
Latency to stand up (s)	424	205	190	252
Latency to first step (s)	460	184	496	157
Number of steps	30	52	10	25
Number of calls	56	67	34	81
Number of defecations	0.6	1.0	0.7	0.8
Frequency of flying	0.3	0.8	0.03	0.2
Social test	6 wk		30 wk	
Number of gentle bouts	0.6	1.3	0.8	1.5
Number of gentle pecks	1.4	3.9	1.5	3.6
Number of severe bouts	0.04	0.29	0.3	0.9
Number of severe pecks	0.09	1.02	0.5	1.5
Number of ground bouts	4.1	5.4	7.3	6.3
Number of aggressive pecks	0.00	0.00	0.14	0.04
Body weight (g)	368	56	1,606	154

[1]Distress call at 5 wk of age, alarm call at 29 wk of age

Heritabilities

Heritability estimates for open-field behaviors at 5 wk of age were high (Table 4). They ranged from 0.20 for the frequency of flying to 0.49 for number of steps. For number of defecations a h^2 of 0.22 was found. In the social test at 6 wk of age, only gentle feather pecking (0.12) and ground pecking (0.13) were found to be heritable behavioral traits. Heritability estimates for severe feather pecking and aggressive pecking and for receiving gentle and severe feather pecking and aggressive pecking were not significantly different from zero. For body weight a h^2 of 0.40 was found. When h^2 were estimated based on either sire or dam variances, h^2 estimates based on the dam component of variance were generally lower than those based on the sire component.

TABLE 4. Heritability (h^2) estimates with standard errors and sire- and dam-based estimates of indicator traits in the open-field test at 5 wk and in the social test at 6 wk of age.

Trait	h^2	SE	h^2_{SIRE}	h^2_{DAM}
Open-field test				
Duration of sitting	0.38*	0.12	0.48	0.26
Duration of standing	0.27*	0.11	0.29	0.20
Latency to first call	0.38*	0.13	0.43	0.23
Latency to stand up	0.35*	0.12	0.46	0.22
Latency to first step	0.45*	0.13	0.58	0.29
Number of steps	0.49*	0.13	0.54	0.45
Number of calls	0.32*	0.11	0.61	0.09
Number of defecations	0.22*	0.09	0.32	0.11
Frequency of flying	0.20*	0.09	0.14	0.19
Social test				
Number of gentle bouts	0.12*	0.07	0.21	0.03
Number of gentle pecks	0.08	0.06	0.14	0.00[1]
Number of severe bouts	0.00	0.02	0.00	0.00
Number of severe pecks	0.02	0.04	0.00[1]	0.05
Number of aggressive pecks	0.02	0.03	0.00	0.00
Number of ground bouts	0.13*	0.07	0.21	0.08
Receiving gentle bouts	0.00	0.00	0.00[1]	0.09
Receiving severe bouts	0.00	0.04	0.00	0.00
Receiving aggressive pecks	0.01	0.03	0.00	0.00
Body weight	0.40*	0.13	0.17	0.34

[1] Estimated sire or dam variance component was negative
*h^2 significantly different from zero

At 29 wk of age, h^2 estimates for behaviors measured in the open-field test were lower than at 5 wk of age, ranging from 0.10 for duration of sitting to 0.20 for latency to first step (Table 5). Heritability estimates of duration of standing, latency to stand up, number of calls, and frequency of flying were not significantly different from zero. In the social test, h^2 were higher compared with 6 wk of age. For gentle feather pecking bouts a h^2 estimate of 0.15 was found, for gentle pecks an estimate of 0.16, and for ground pecking an estimate of 0.30. Also at 30 wk of age, h^2 estimates for severe feather pecking and aggressive pecking and for receiving gentle and severe feather pecking and aggressive pecking were not significantly different from zero. Comparable with the results from 5 and 6 wk of age, h^2 estimates based on the dam component of variance were generally lower than those based on the sire component when h^2 were estimated based on either sire or dam variances.

TABLE 5. Heritability (h^2) estimates with standard errors and sire- and dam-based estimates of indicator traits in the open-field test at 29 wk and in the social test at 30 wk of age.

Trait	h^2	SE	h^2_{SIRE}	h^2_{DAM}
Open-field test				
Duration of sitting	0.10*	0.06	0.23	0.05
Duration of standing	0.08	0.05	0.23	0.03
Latency to first call	0.18*	0.09	0.43	0.09
Latency to stand up	0.07	0.05	0.11	0.08
Latency to first step	0.20*	0.09	0.28	0.18
Number of steps	0.15*	0.08	0.09	0.21
Number of calls	0.09	0.06	0.19	0.00
Number of defecations	0.16*	0.08	0.22	0.11
Frequency of flying	0.04	0.04	0.03	0.05
Social test				
Number of gentle bouts	0.15*	0.08	0.27	0.14
Number of gentle pecks	0.16*	0.08	0.23	0.19
Number of severe bouts	0.06	0.05	0.07	0.02
Number of severe pecks	0.07	0.05	0.09	0.07
Number of aggressive pecks	0.01	0.03	0.05	0.00[1]
Number of ground bouts	0.30*	0.20	0.28	0.20
Receiving gentle bouts	0.04	0.06	0.00[1]	0.19
Receiving severe bouts	0.00	0.03	0.00	0.00
Receiving aggressive pecks	0.03	0.04	0.06	0.00[1]
Body weight	0.50*	0.14	0.75	0.35

[1] Estimated sire or dam variance component was negative
*h^2 significantly different from zero

DISCUSSION

The aim of the current experiment was to estimate h^2 of feather pecking and open-field response in young and adult birds. High h^2 were found for open-field behaviors at 5 wk of age. In the social test at 6 wk of age, gentle feather pecking and ground pecking were found to be heritable. At 29 wk of age, h^2 of open-field behaviors were lower than at 5 wk of age. For gentle feather pecking and ground pecking higher h^2 were found at 30 wk of age compared with 6 wk of age. The h^2 of severe feather pecking was not significantly different from zero at either age.

Heritability Estimates

In the present study h^2 were estimated in an F_2 population. This may affect the estimates, as the variation in the F_1 population is the mean of variation within the original lines (Lande, 1981). If the lines to make the cross would have been inbred strains, the

variance between families would be zero. Although inbred strains were not used in the current study, population structure may have affected the estimates. Also the distributions of the traits have to be taken into account. Most distributions of behavioral traits were skewed to the right with many observations with value zero. When h^2 were estimated based on transformed frequencies (square root transformation) and latencies (log transformation), however, estimates were comparable with the estimates based on the non-transformed data, while the distribution of the transformed traits was closer to a relatively normal distribution. Finally, the number of animals used in this experiment was limited compared with studies where h^2 for production traits were estimated. For a behavioral study, however, the current study on 630 individual birds was large and the number of studies on populations of this size is limited. This is mainly due to the labor intensity of behavioral observations at individual level.

Heritabilities Open-Field Test

At 5 wk of age, h^2 for locomotion traits in the open-field were high. The h^2 of 0.49 for number of steps is in close agreement with the h^2 found by Boyer et al. (1970) for overall movement. The estimated h^2 for number of defecations of 0.22 is higher than those reported by Boyer et al. (1970) and Faure (1981) of 0.06 and 0.10 respectively. At 29 wk of age, h^2 for open-field behaviors were much lower than at 5 wk of age, comparable with the results found by Webster and Hurnik (1989) in 17 wk old pullets. Most of the open-field tests in poultry have been performed with young chicks (Boyer et al., 1970; Gallup and Suarez, 1980; Faure, 1981; Jones et al., 1995). In chicks, the motivation to return to their flock-mates is strong, as is their fear of being detected by predators. In adult laying hens, these motivations may be less strong, as they seem to be more important to young and vulnerable birds.

Heritabilities Social Test

In the social test at both 6 and 30 wk of age, only gentle feather pecking, ground pecking, and body weight were found to be heritable traits, with higher h^2 at 30 wk of age. Kjaer and Sørensen (1997) also found higher h^2 for feather pecking at 38 wk of age compared with 6 wk of age. The h^2 estimates for gentle feather pecking of 0.12 at 6 wk of age and 0.15 at 30 wk of age fit well with the estimates for total feather pecking in previous studies (Cuthbertson, 1980; Bessei, 1984; Kjaer and Sørensen, 1997). The h^2 of severe feather pecking was not significantly different from zero at both ages. At 6 wk of age, only 14 out of 630 birds performed severe feather pecking, so the low incidence may be part of the

explanation. At 30 wk of age, however, the incidence was higher and yet the same result was found. This indicates that, under the conditions used in this experiment, the development of severe feather pecking depends mainly on a combination of environmental factors and not so much on genetic factors. In previous studies, no distinction was made between gentle and severe feather pecking, but the present results indicate that it may useful to separate them, as they seem to be controlled by different mechanisms. Heritabilities of receiving gentle or severe feather pecks or aggressive pecks were not significantly different from zero in the present study. In the case of receiving severe feather pecking or aggressive pecking the low incidence may play a role. Kjaer and Sørensen (1997) found a low h^2 (0.15 ± 0.07) for receiving feather pecking at 6 wk of age.

Sire- and Dam-Based Heritabilities

When h^2 were estimated based on either sire or dam variances, in most cases the sire-based estimates were considerably higher than the dam-based estimates. These results were against expectations, since any presence of dominance and maternal effects would be included in the dam component of variance. Maternal genetic effects have, for instance, been shown to affect body weight and ascites-related traits in broilers (Pakdel et al., 2002). In the present study, the h^2 estimate for body weight based on the dam variance is higher than the estimate based on the sire variance at 6 wk of age, but lower at 30 wk of age. The standard errors of the estimates of the sire- and dam-based h^2 were higher than those of the ASREML estimates, which may also explain the apparent differences in sire- and dam-based h^2. Genetic causes may provide an alternative explanation, with possible explanations being sex linkage or parent-of-origin effects. It was not possible to investigate this further from the available data.

In conclusion, gentle feather pecking and open-field response were found to be heritable, which may offer the possibility for genetic selection against feather pecking in the future. For open-field behaviors h^2 were higher at 5 wk of age compared with 29 wk of age. For gentle feather pecking, ground pecking and body weight, measured in the social test h^2 were higher at 30 wk of age than at 6 wk of age. The h^2 estimate for severe feather pecking was not significantly different from zero at either age. In further research, the relationships between feather pecking and open-field response will be studied to identify possible predictors of feather pecking, supplying tools to select against feather pecking.

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CHAPTER 3

Mapping Quantitative Trait Loci Affecting Feather Pecking Behavior and Stress Response in Laying Hens

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ABSTRACT

In the European Union, legislation concerning animal housing is becoming more strict, because of animal welfare concerns. Feather pecking (FP) in large group housing systems is a major problem. It has been suggested that corticosterone (CORT) response to manual restraint as a measure for stress is associated with FP behavior. The aim of the current study was to identify QTL involved in FP behavior and stress response in laying hens. An F_2 population of 630 hens was created from a cross between two commercial lines of laying hens differing in their propensity to feather peck. The behavioral traits measured at 6 and 30 wk of age were: gentle FP, severe FP, and aggressive pecking. Toe pecking was measured at 30 wk of age and CORT response to manual restraint was measured at 32 wk. All animals were genotyped for 180 micro-satellite markers. A QTL analysis was performed using a regression interval mapping method. At 6 wk of age a suggestive QTL on GGA10 was detected for gentle FP. At 30 wk of age suggestive QTL were detected on GGA1 and GGA2 for gentle FP. A significant QTL was detected on GGA2 for severe FP. At 32 wk of age a suggestive QTL was detected on GGA18 for CORT response to manual restraint. In addition, a suggestive QTL was detected on GGA5 with possible maternal parent-of-origin effect for CORT response.

(*Key words:* quantitative trait locus, feather pecking, behavior, chicken, stress response)

INTRODUCTION

In the European Union, legislation concerning animal housing is becoming more strict, because there is increasing concern for animal welfare. There is a shift from individual housing systems to large group housing systems in poultry management. Feather pecking (FP) in large group housing systems is a major problem (Blokhuis et al., 2000). The FP is characterized as pecking at the plumage of another bird. There are different forms of FP behavior ranging from gentle FP to severe FP. This behavior results in denuded areas and wounds and can ultimately result in cannibalistic behavior (Savory, 1995). The damage increased with the age of the chickens.

It would be of interest to have a predictor for FP already at early age. Recent studies show that there is a positive correlation between FP behavior at young age and at adult age (Kjaer and Sørensen, 1997). Consequently, gentle FP behavior observed at young age might be a useful predictor for gentle FP behavior at an adult age. Severe FP, however, at a young age is not a useful predictor for severe FP at an adult age. Severe FP is the most damaging form of FP (Savory, 1995).

Genetics may give new possibilities to reduce the FP problem, since it has been shown that there is genetic variation for FP behavior. The heritability for FP is in the range of 0.05 to 0.50 (Cutbertson, 1980; Bessei, 1984; Kjaer and Sørensen, 1997). In addition, it has been shown that selection on cannibalistic behavior using group selection (Muir, 1996) or selection on FP behavior using direct observations (Kjaer et al., 2001) is feasible.

Physiological characterization of a high feather pecking (HFP) and low feather pecking (LFP) line described by Blokhuis and Beutler (1992) showed that these lines differ in acute corticosterone (CORT) response to a manual restraint test (Korte et al., 1997; Van Hierden et al. 2002). The HFP line is characterized by a low CORT response and active behavioral reaction to a stressor, while, in contrast, the LFP line showed the opposite characteristics. In the study by Korte et al. (1997), it was hypothesized that FP represents a failure of normal adaptive mechanisms to operate, and that glucocorticoids play an important role in that process via the mineralocorticoid receptor-glucocorticoid receptor balance.

The use of molecular genetics can facilitate the search for the molecular basis of FP behavior and stress response. In chickens, the tools to dissect the molecular basis of a quantitative traits, such as the chicken consensus genetic linkage map (Groenen et al., 2000), and the chicken bacterial artificial chromosome library (Crooijmans et al., 2000) are available.

In addition, sequencing of the chicken genome was started in 2002 (http://genome.gov/page.cfm?pageID=10002154). The availability of the chicken genome sequence will be of great help in the identification of genes in the QTL region. The aim of the current study was to identify QTL involved in pecking behavior at young and adult age and in stress response at an adult age in an F_2 population originating from a cross between the HFP and LFP lines.

MATERIALS AND METHODS

Experimental Population

An F_2 population was created from a cross between two lines of laying hens. The HFP and LFP lines differ for behavioral traits (Blokhuis and Beutler, 1992; Jones et al., 1995) as well as for physiological traits (Korte et al., 1997; Van Hierden et al., 2002). Six males from the HFP line were mated to 6 females from the LFP line and 6 males from the LFP line were mated to 6 females from the HFP line to generate 120 F_1 animals. Seven F_1 males were mated to 28 F_1 female birds to produce 630 F_2 hens. On average there were 90 progeny per sire and 23 progeny per dam. The F_2 hens arrived at the experimental farm as day-old-chicks in 5 batches at 2 wk intervals. The birds were not beak-trimmed and each individual bird was marked with a wing-band. Each batch was divided over 2 pens, giving a total of 10 groups (batch x pen) with an average of 63 birds per group. The floor area of the pen was 4.75 x 2 m and covered with wood-shavings. There were 2 light tubes (2 x 40W) in each pen, and during wk 0 to 4 a heating lamp was provided. From wk 0 to 4 continuous light provided by the heating lamp, while in wk 5 to 6 the scheme was changed to 8 h light per day from 0800 to 1600 h. From 16 wk of age onwards the light scheme was extended 1 h per week until the animals had a 16-h light day from 0300 to 1900 h. Feed (152 g/kg crude protein and 2,817 kcal/kg metabolizable energy) and water was provided for ad libitum consumption.

Phenotypic Data

At 6 wk of age, 625 F_2 birds and, at 30 wk of age, 550 F_2 birds were tested using a social FP test (Rodenburg and Koene, 2003). For this test, five birds were randomly picked from their home pen and transferred to a testing pen in a sound attenuated room. Birds in one test group were from the same batch and home pen. The testing pen was an square open-field

of 1.25 x 1.25 m with wood shavings on the floor. During the test, the birds had no access to feed, water, perches, and laying nests in the testing pen. The birds were placed in the testing pen in darkness. Observations started when the light (2 tubes of 40 W each) was switched on. The FP behavior was directly recorded from an adjacent observation room using a video camera.

The traits measured at 6 and 30 wk were: gentle FP (gentle pecks, ignored by recipient), severe FP (forceful peck, reaction of the receiver), and aggressive pecking (forceful peck aimed at the head or neck). In addition, at 30 wk toe pecking (forceful peck aimed at the toe or leg) was measured. The number of pecks and the number of bouts, a period of continuous pecking directed towards the same part of the body of the conspecific, were recorded for each trait. A detailed description concerning the distribution, averages, and standard deviation of the traits were presented in an earlier paper (Rodenburg et al., 2003).

After the FP test at 30 wk, the animals were housed individually. At 32 wk of age, the hens (n=524) were exposed to a manual restraint test. The test was performed between 0900 and 1200 h. For this test, the bird was placed on its side for 8 min. A blood sample (1mL) was taken from the wing vein after 8 min of manual restraint. Blood samples were transferred to heparine-coated centrifuge tubes and chilled on ice (0 °C) and centrifuged at 3,000 rpm for 10 min at 4 °C. The supernatant was stored at 4 °C until analysis. The CORT concentration (ng/mL) was measured in duplicate (De Jong et al., 2001). Average values of the two CORT samples were used in the analysis. The Wageningen University Committee on Animal Care and Use has approved the use of the birds in the current experiment.

Genotypic Data

Blood was taken from the wing vein from 5 wk old birds and DNA was extracted according to the Capture Plate^{TM} Kit protocol[1]. All birds from the F_0, F_1, and F_2 generation were genotyped with 180 micro-satellite markers. These markers were covering GGA1-GGA19, GGA23, GGA24, GGA27, GGA28, GGAZ, and linkage groups E38, E47W24, E60E04W23 (Groenen et al., 2000). Markers were labeled with a fluorescent dye (6-FAM, HEX, or TET). The amplification reactions were performed as described by Crooijmans et al. (1997). The PCR program used was: 5 min denaturation at 95 °C, 36 cycles of 30 s at 95 °C, 30 s at annealing temperature, and 30 s at 72 °C followed by a final elongation step of 4 min

[1] Gentra Systems, Minneapolis, MN, 55447

at 72 °C. Markers were divided over 13 sets based on their fragment size and run on an ABI373 sequencer[2] as described by Crooijmans et al. (1997). Fragment sizes were calculated relative to the GENESCAN-350 TAMRA marker with GENESCAN 2.1 fragment analysis software[2] and allele identification was performed using GENOTYPER 2.0 software[2]. All genotypic data were checked by two independent individuals prior to inheritance checking using CRI-MAP (Green et al., 1990).

Genome-Wide Scan

Prior to the genome scan analysis, behavioral observations at 6 wk were adjusted using the PROC GLM procedure (SAS Institute, 1985) with testgroup (j = 1,2,....,129) (group in which the birds were tested) as a fixed effect in the model. Also at 30 wk of age behavioral observations were adjusted with testgroup (j = 1,2,...,112) as a fixed effect in the model. No significant effect of homepen (pen in which the bird was housed) or batch (order in which the birds arrived at the farm) were found on the behavioral traits. The data on CORT response to manual restraint was adjusted for batch (j = 1,2,...,5) as a fixed effect in the model.

A regression method was used for interval mapping. Two different genetic models were used: 1) paternal half-sib analysis (Knott et al., 1996; De Koning et al., 1999) and 2) line-cross analysis (Haley et al., 1994; De Koning et al., 2000). In the paternal half-sib model, no assumptions were made concerning the allele frequencies in the founder lines or the number of QTL alleles. The F_2 animals were treated as 7 unrelated half-sib families using the model:

$$Y_{ij} = m_i + b_i p_{ij} + e_{ij}$$

where Y_{ij} = trait measured on animal j, from rooster i; m_i = average of half-sib family i; b_i = substitution effect for a putative QTL; p_{ij} = the conditional probability for animal j of rooster i inheriting the first paternal haplotype; e_{ij} = residual effect. In this analysis the contrast between the two haplotypes of every F_1 rooster was made. Analyses within families were performed and the test statistics were calculated as an F ratio for every centi Morgan on the chromosome (De Koning et al., 1999).

[2] Applied Biosystems, Perkin-Elmer, Foster City, CA 94404

In the line-cross model the alternative alleles at the QTL were traced back to the founder lines. De Koning et al. (2000) have adapted the model for the detection of parent-of -origin effects containing a paternal, a maternal, and a dominance component:

$$Y_j = m + a_{pat}p_{patj} + a_{mat}p_{matj} + dp_{dj} + e_j$$

where Y_j = trait measured on animal j; m = population mean; a = additive effect; d = dominance effect; p_{pat} = conditional probability that an animal inherited a HFP allele from the sire ($[p_{11} + p_{12}] - [p_{22} + p_{21}]$); p_{mat} = conditional probability that an animal inherited a HFP allele from the dam ($[p_{11} + p_{21}] - [p_{22} + p_{12}]$).

The threshold levels were empirically derived using a permutation test (Churchill and Doerge, 1994). The threshold was determined using 10,000 permutations. The threshold levels as suggested by Lander and Kruglyak (1995) were used: 1) chromosome wide linkage (multiple testing across one chromosome is accounted for); 2) suggestive linkage (statistical evidence expected to occur one time at random in a genome scan); and 3) significant linkage (statistical evidence expected to occur 0.05 times in a genome scan).

For QTL with possible parent-of-origin effect an additional test of the full model against the mendelian model was performed. The genome was screened for imprinted QTL using an imprinted model (either maternally or paternally). At locations where significant evidence for the presence of an imprinted QTL was found, it was tested if an imprinted QTL explained the observations better than a mendelian model (Knott et al., 1998). The test used was an F test with 1 d.f. in the numerator and (n-4) d.f. in the denominator. The test of Knott et al. (1998) is, in general, somewhat more conservative than the test proposed by De Koning et al. (2000) as reported in De Koning et al. (2002).

RESULTS

Genotyping

The grand parents were tested for polymorphism using 180 micro-satellite markers. Twenty-eight markers could not be used in the genome wide scan either because the markers did not amplify, or the markers were not informative in the cross. The map distances based on 152 micro-satellite markers, in general, did not differ much from the distances on the consensus linkage map, therefore the map distances based on the consensus map were used. An exception were the markers MCW0370 and MCW0371 on GGA16. Although the physical distance between the markers is 1,882 bp (accession number: AL023516) the estimated mapping distance in the current data was 20 centi Morgan in a two-point linkage analysis. Three independent persons checked the data on GGA16; however, no conclusive answer can be given whether this was a natural occurring phenomenon or a typing error. The estimated genome coverage was approximately 80%. For the markers on GGA16, E38, and E47W24, a genotype could not be established in the F_0 animals. In the line-cross analysis, the contrast could not be estimated between the HFP and the LFP alleles, because the alleles can not be traced from the F_0 to the F_2 animals.

Half-Sib Analysis

The results found for the behavior traits of pecking behavior at 6 wk of age using the half-sib analysis are presented in Table 1. For gentle FP (number of bouts), a QTL exceeding the 5% chromosome wide threshold was detected, on GGA10, GGA17, and GGA24. For GGA10, family 2 had a QTL allele substitution effect of -1.58 and contributed most to the overall test statistics. For GGA17, family 6 had a QTL allele substitution effect of -2.23 and contributed most to the overall test statistics with an F ratio of 26.1. For GGA24, family 2 had a QTL allele substitution effect of 1.05 and an F ratio of 5.4. For number of pecks (gentle FP), one QTL region exceeding the 5% chromosome wide threshold on GGA24 was found. Family 2 had a QTL allele substitution effect of 2.86 and an F ratio of 7.0. For the traits severe FP and aggressive pecking, no QTL were detected using the half-sib analysis.

TABLE 1. QTL affecting pecking traits and stress response using the half-sib analysis.

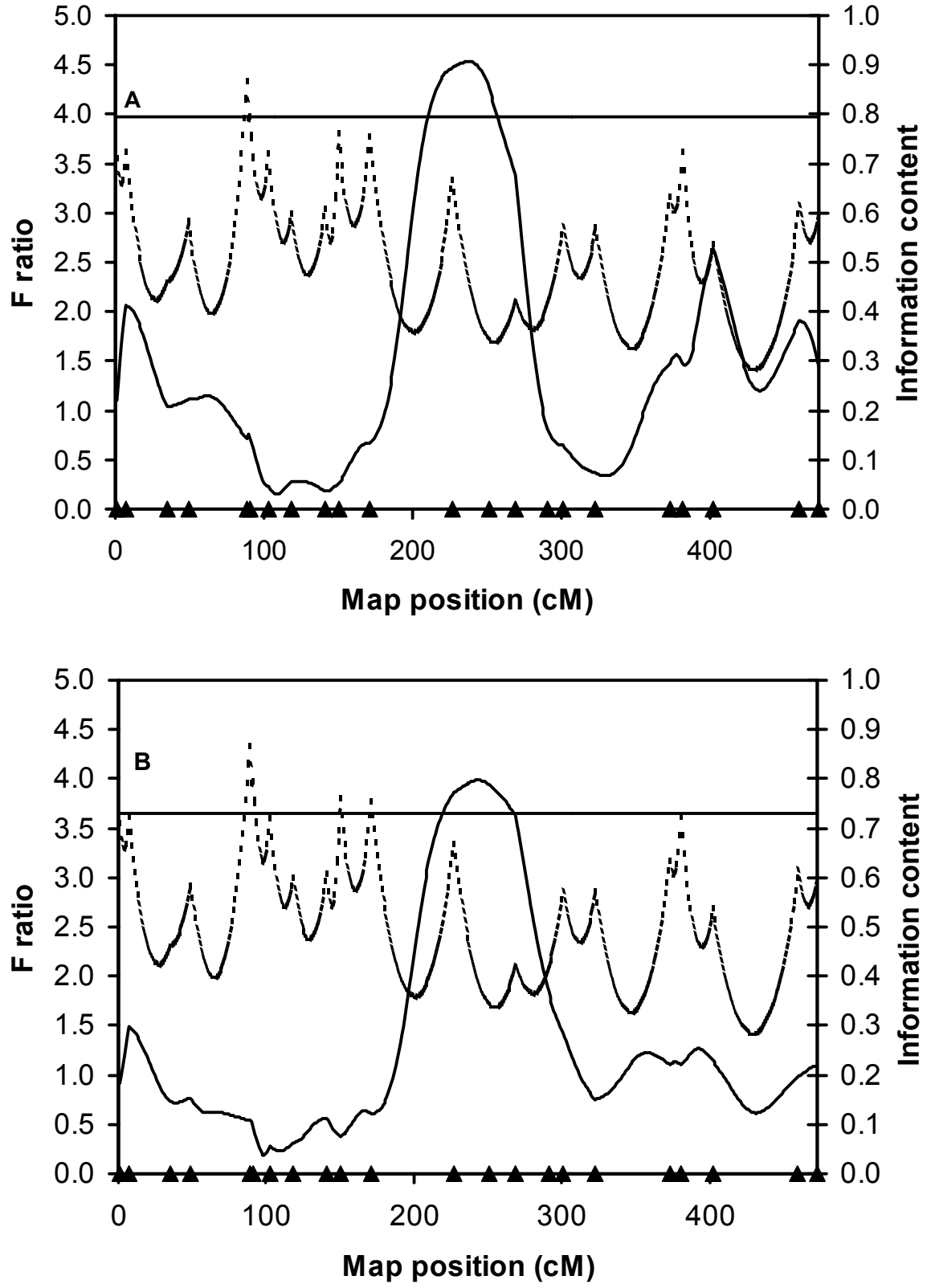
Trait	Chromosome	Position (cM)	Marker bracket	F ratio[1]
6 wk				
Gentle FP[2] (no. bouts)	GGA10	25	MCW0228-ADL0209	3.24‡
	GGA17	1	ADL0149-MCW0330	3.53‡
	GGA24	1	MCW0301-LEI0069	2.22‡
Gentle FP[2] (no. pecks)	GGA24	1	MCW0301-LEI0069	2.64‡
Aggressive pecking	-			
30 wk				
Gentle FP[2] (no. bouts)	GGA2	243	MCW0042-MCW0087	3.98†
Gentle FP[2] (no. pecks)	GGA2	237	MCW0042-MCW0087	4.54†
Aggressive pecking	GGA12	1	ADL0372-MCW0198	3.42‡
32 wk				
CORT[3]	GGA18	33	ROS0022-MCW0219	3.12†

[1] ‡chromosome wide linkage; †suggestive linkage.
[2]FP = feather pecking.
[3]CORT = corticosterone response (ng/mL) after a manual restraint test as measurement for acute stress response.

The results found for the behavior traits at 30 wk of age and the stress response using the half-sib analysis are presented in Table 1. For 'number of bouts' a suggestive QTL was detected on GGA2 between marker brackets MCW0042 and MCW0087 (Figure 1). Family 5 with a QTL allele substitution effect of 3.62 and an F ratio of 6.48 and family 6 with a QTL allele substitution effect of -2.55 contributed most to the pooled test statistics. For aggressive pecking, a chromosome wise significant QTL was detected on GGA12. For gentle FP a suggestive QTL was detected on GGA2 for 'number of pecks' between marker brackets MCW0042 and MCW0087 (Figure 1). The contribution to the QTL came from two families. Family 5 with a QTL allele substitution effect of 1.78 and an F ratio of 7.49. Family 6 had a QTL allele substitution effect of -0.93 and an F ratio of 3.19. For severe FP and toe pecking, no QTL were detected under the half-sib analysis.

For CORT response after manual restraint, a suggestive QTL was detected on GGA18 (Table 1). The contribution to the QTL for CORT was mainly coming from family 2, which had a QTL allele substitution effect of -1.95 and an F ratio of 12.94.

FIGURE 1. QTL for gentle feather pecking on GGA2 under the half-sib analysis. A = number of pecks. B = number of Bouts. The F statistics profile is given as a solid line. The information content is given as a dashed line. The horizontal line refers to the suggestive significance level. Marker positions on the chromosome are indicated as triangles.



Line-Cross Analysis

The results found for the behavior traits for pecking behavior at 6 wk of age using the line-cross analysis are presented in Table 2. A suggestive QTL on GGA10 for gentle FP was detected between marker brackets ADL0209 and MCW0067 for the trait 'number of bouts' as well as for the trait ‘number of pecks’. The QTL on GGA10 for ‘number of bouts’ was mainly additive in nature and had a negative additive effect, indicating that the segment coming from the HFP line had a negative effect on performing gentle FP (Table 2). The QTL on GGA10 for ‘number of pecks’ had a negative additive effect, and there was some evidence for the presence of dominance. Using the maternal parent-of-origin model, a genome-wide significant QTL with maternal parent-of-origin effect for gentle FP were detected on GGA10 for the traits 'number of bouts' and 'number of pecks' when tested for parent-of-origin effects against the null hypothesis of no QTL. However, the test of the full model against the mendelian model was not significant, indicating that the QTL on GGA10 was mendelian in nature.

The results found for the behavior traits for pecking behavior at 30 wk of age using the line-cross analysis are presented in Table 2. A suggestive QTL was detected on GGA1 for gentle FP (number of pecks) between marker brackets ADL0068 and LEI0146. A chromosome wide QTL on GGA15 was detected between marker brackets MCW0052 and MCW0080. For gentle FP using ‘number of bouts’ as a trait no QTL was detected. When testing for parent-of-origin effects against the null hypothesis of no QTL for gentle FP ‘number of bouts’, two suggestive paternally imprinted QTL on GGA8 and GGA24 were identified. However the test of the full model against the mendelian model did not provide statistical evidence for parent-of-origin effects. Using gentle FP ‘number of pecks’ as a trait, a suggestive QTL with maternal parent-of-origin effect was detected, when tested against the null hypothesis of no QTL. The test of the full model against the mendelian model did not provide statistical evidence for parent-of-origin effects.

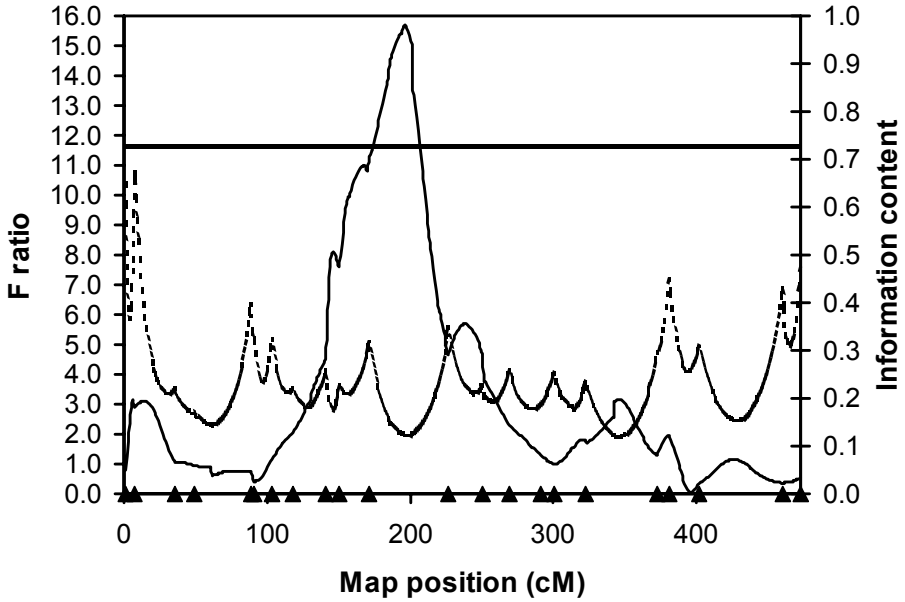
A significant QTL for severe FP using ‘number of bouts’ as a trait was detected on GGA2 between marker brackets ABR0008 and MCW0042 (Figure 2).

TABLE 2. QTL affecting pecking traits and stress response using the line-cross analysis.

Trait	Chromosome	a (SE)[1]	d (SE)[1]	Position (cM)	Marker bracket	F ratio[3]
6 wk						
Gentle FP[2] (no. bouts)	GGA10	-1.14 (0.29)	-0.04 (0.62)	53	ADL0209-MCW0067	7.53†
	GGA24	0.72 (0.26)	-1.13 (0.53)	8	MCW0301-LEI0069	5.87‡
Gentle FP[2] (no. pecks)	GGA10	-3.87 (0.99)	-3.64 (2.00)	48	ADL0209-MCW0067	9.99†
	GGA11	2.82 (1.16)	-6.92 (2.74)	63	ADL0210-ADL0308	5.61‡
	GGA24	2.88 (0.89)	-3.00 (1.85)	8	MCW0301-LEI0069	6.39‡
30 wk						
Gentle FP[2] (no. pecks)	GGA1	2.46 (0.78)	-5.27 (1.86)	134	ADL0068-LEI0146	10.37†
	GGA15	1.23 (0.69)	-4.28 (1.48)	39	MCW0052-MCW0080	5.33‡
Severe FP[2] (no. bout)	GGA2	-1.83 (0.40)	-4.16 (0.97)	196	ABR0008-GCT0020	15.71*
	GGA11	0.31 (0.31)	-3.21 (0.96)	37	ADL0023-ADL0210	6.62‡
Severe FP[2] (no. pecks)	GGA2	-3.52 (0.85)	-5.41 (2.02)	195	ABR0008-GCT0020	10.38*
Aggressive pecking	GGA12	-2.92 (1.23)	-14.58 (3.84)	33	ADL0372-MCW0198	8.99‡
32 wk						
CORT[4]	GGA15	-0.40 (0.16)	0.69 (0.32)	35	MCW0231-MCW0080	4.69‡

[1]Estimated QTL effects for the genetic model. a is the additive effect; d is the dominance level. Standard errors are in parenthesis.
[2]FP = feather pecking .
[3] ‡chromosome wide linkage; †suggestive linkage; *genome wide linkage at $P < 0.05$.
[4]CORT = corticosterone response (ng/mL) after a manual restraint test as measurement for acute stress response

FIGURE 2. QTL for severe feather pecking ('number of Bouts') on GGA2 under the line-cross analysis. The F-statistics profile is given as a solid line. The information content is given as a dashed line. The horizontal line refers to the 5% genome wide significance level. Marker positions on the chromosome are indicated as triangles



In addition, a chromosome wide QTL was detected on GGA11 between marker brackets ADL0023 and ADL0210. No QTL was detected when testing for parent-of origin effects. For severe FP recorded as 'number of pecks', a significant mendelian QTL on GGA2 was detected between marker brackets ABR0008 and MCW0042. The allele coming from the HFP line had a decreasing effect on the number of bouts for severe FP. For aggressive pecking, a chromosome wide QTL was detected on GGA12 between marker brackets ADL0372 and MCW0198. The allele coming from the HFP line had a decreasing effect on aggressive pecking behavior. For toe pecking no QTL was detected using the line cross analysis.

For CORT response to manual restraint, a chromosome wide QTL was detected on GGA15, between marker brackets MCW0231 and MCW0080 (Table 2). When testing for parent-of-origin effects, two suggestive QTL on GGA1 and GGA5 were detected. When testing the full model against the mendelian model only the QTL on GGA5 was significant, indicating that the full model explained more variance than the mendelian model. When testing the components (paternal, maternal, and dominance) in the full model of the QTL on GGA5 separately, only the paternal component was significant. The suggestive QTL with a maternal parent-of-origin effect had a highest F value between marker brackets MCW0038 and MCW0214 and an additive effect (SE) of 0.39 (0.13).

DISCUSSION

The QTL mapping was a helpful tool to find the underlying genes for complex traits, like behavior. In the current study, it is shown that it is possible to identify QTL involved in FP behavior in laying hens at two different ages. In addition, a QTL was detected that was involved in the CORT response to manual restraint as a measure for stress response.

The HFP and the LFP lines used in the present study have been selected on production traits and not on behavioral traits; therefore, alleles affecting behavioral traits might be segregating in the two populations. However, when the difference in pecking behavior is a consequence of drift or co-selection, QTL alleles for behavior traits might be fixed in the founder lines. Therefore, two different models were used in the QTL analysis: the half-sib analysis and the line-cross analysis. The assumptions concerning allele frequencies in the founder lines and the family structure are different between the half-sib and line-cross model (De Koning et al., 1999, 2000). The half-sib model does not make a-priori assumptions for the QTL alleles in the founder lines, while, under the line-cross model, the alternative QTL alleles are traced back to the founder lines. Using both QTL detection methods, partly different QTL were identified under the half-sib model and line-cross model. This result indicated that QTL alleles involved in pecking behavior were segregating in the founder lines. In general, it is assumed that the line-cross model is more powerful to detect QTL. This assumption, however, is only valid when the QTL alleles are indeed fixed in the founder lines (Alfonso and Haley, 1998). The power to detect a QTL rapidly decreases if the allele frequencies for the trait of interest are not fixed in the founder lines (De Koning et al., 2002).

Assuming the difference in FP behavior between the LFP and HFP was due to co-selection of egg production traits, one might expect to find overlapping QTL for both traits. Tuiskula-Haavisto et al., (2002) identified QTL for egg production traits on GGA2. The QTL on GGA2 for severe FP and gentle FP identified in the current study, however, were more than 100 centi Morgan apart. Therefore, it is not likely that there are pleiotrophic or closely linked genes involved in the control of FP behavior and egg production traits on GGA2.

Feather pecking is mainly a problem in adult laying hens. To be able to select for FP behavior, good criteria have to be defined in how to quantify FP. In addition, it would be useful to have a reliable predictor for this phenomenon at a young age to be able to cull the birds which are likely to be the peckers in an early stage. Gentle FP was recorded as 'number of bouts' and 'number of pecks' (Rodenburg et al., 2003). The observation 'number of bouts'

and 'number of pecks' are highly correlated (Kjaer and Sørensen, 1997). Therefore, it is not surprising that similar QTL profiles were found for gentle FP on GGA10 for a young age and on GGA2 for the adult age and for severe FP on GGA2 observed as bouts and pecks. The results indicated that for practical observations and selection on FP behavior, one could use either bouts or pecks as selection criterion. However, the age of measuring FP behavior for selection is important. Although Kjaer and Sørensen (1997) found a positive correlation between ages for gentle FP behavior, T. B. Rodenburg (Wageningen University, Wageningen, The Netherlands, personal communications) did not find a correlation between ages for gentle FP. The QTL detected in the current study for gentle FP at a young age did not coincide with QTL for gentle FP at adult age. Although it is still possible that coinciding QTL did exist between ages, the results indicate that gentle FP is regulated by different QTL at different ages. As a consequence, gentle FP at young age is not a predictor for FP in adult hens.

From the present study it is clear that it is possible to detect QTL for gentle FP; however, the focus should be on severe FP because severe FP is a form of FP that causes the most damage (Savory, 1995). A significant QTL on GGA2 was identified using the line-cross analysis. Although there is a correlation between gentle FP and severe FP at the same age (T. B. Rodenburg, Wageningen University, Wageningen, The Netherlands, personal communications), it is likely that these QTL were not the same, because these QTL have been detected under different genetic models. However, based on the data available it was not possible to exclude the possibility of overlap between the QTL found. Due to the stricter legislation in the European Union concerning animal welfare, improvement of animal welfare becomes more and more important. Further analysis of this QTL region will help to identify candidate genes involved in severe FP. The identification of candidate genes or genetic markers related to severe FP opens the prospective to prevent the FP problem in commercial flocks using molecular genetic approaches.

Care should be taken in the interpretation of the presented parent-of-origin QTL on GGA5 for CORT response to manual restraint. According to the imprinting theory of Moore and Haig (1991), this phenomenon does not occur in birds. To date, there is no unambiguous evidence that parent-of-origin effects do or do not exist in chickens. In chickens, there are some reports indicating reciprocal effects for production traits (Fairfull et al., 1983; Wearden et al., 1965). Bessei (1984) produced a reciprocal cross between a Rhode Island Red and a Sussex line. There was a difference in FP between the pure lines, the reciprocal crosses, however, were intermediate. To date, clear biological evidence concerning parent-of-origin

effects in chicken has not been found. Only a few papers report on the allelic expression in chicken embryos in which the focus is mainly on the IGF2 gene (O'Neill et al., 2000; Koski et al., 2000; Nolan et al., 2001; Yokamine et al., 2001). Koski et al. (2000) stated that they found mono-allelic expression in chicken embryos; however, this was not confirmed by others (O'Neill et al., 2000; Nolan et al., 2001; Yokamine et al., 2001). Based on the literature, there is no conclusive evidence that parent-of-origin effects do or do not exist in chickens. The actual biological evidence for parent-of-origin effect in poultry should come from expression studies at the RNA and protein level. Nevertheless, the observation of parent-of-origin effects are interesting and deserve more detailed analysis.

Corticosteroids are important in immune regulation (Bauer et al., 2001), metabolic pathways, and adaptive behavior (Korte, 2001). In mammals, behavioral response to stressors is associated with differences in CORT response. Animals could be assigned to groups according to their coping strategy either to the pro-active group or to the reactive group (Koolhaas et al., 1999). Pro-active animals have an active behavioral response and a low adrenocortical response to a stressor. Reactive animals have an inactive behavioral response and a high adrenocortical response (Koolhaas et al., 1999). Korte et al. (1997) suggested that the LFP and HFP line are representatives of, respectively, the reactive and pro-active coping style (Korte et al., 1997; Van Hierden et al., 2002). The direct relation between CORT response and FP behavior, however, is not clear. Although feather pecking may be associated with stress (El-lethy et al., 2001), in the current study, there was no indication that CORT response to manual restraint showed coinciding QTL with the FP behavioral traits. So far, the relation between CORT response and feather pecking was studied on a line level (Korte et al., 1997; Van Hierden et al., 2002). However, this should also be studied at the individual animal level.

The current identification of QTL involved in FP behavior open the possibility to dissect the genetic basis of FP behavior. Identification of genes involved in behavioral traits could facilitate breeding companies to select more specifically on welfare-related traits. The free-range housing systems requires chickens which have a lower tendency to feather peck to avoid mortality due to pecking incidences. Further fine-mapping of the QTL region using, e.g., subsequent generations and improvement of the comparative map in the QTL regions will provide information on possible candidate genes.

In the present study, QTL were detected for severe FP, gentle FP, and CORT response. The results indicated that FP behavior at 6 wk of age is regulated by different genes

than FP at 30 wk of age. Furthermore, the results opens the possibility to reduce the FP problem and improving animal welfare using molecular genetics.

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CHAPTER 4

Improvement of the comparative map of chicken chromosome 13

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ABSTRACT

A comparative map was made of chicken chromosome 13 (GGA13) with a part of human chromosome 5 (HSA5). Microsatellite markers specific for GGA13 were used to screen the Wageningen chicken bacterial artificial chromosome (BAC) library. Selected BAC clones were end sequenced and 57 sequence tag site (STS) markers were designed for contig building. In total, 204 BAC clones were identified which resulted in a coverage of about 20% of GGA13. Identification of genes was performed by a bi-directional approach. The first approach starting with sequencing mapped chicken BAC subclones, where sequences were used to identify orthologous genes in human and mouse by a basic local alignment search tool (BLAST) database search. The second approach started with the identification of chicken orthologues of human genes in the HSA5q23-35 region. The chicken orthologous genes were subsequently mapped by fluorescent *in situ* hybridization (FISH) and/or single nucleotide polymorphism typing. The total number of genes mapped on GGA13 is increased with 14 to a total of 20 genes. Genes mapped on GGA13 have their orthologues on HSA5q23-5q35 in human and on Mmu11, Mmu13 Mmu18 in mouse.

(Key words: chicken, comparative mapping, single nucleotide polymorphism)

INTRODUCTION

Completion of the sequencing of the human genome and the ongoing efforts for the mouse genome provide an efficient tool for comparative mapping in other species. The main focus of comparative mapping is between mammalian species, e.g. rat - mouse - Human (Gauguier *et al.* 1999) and Cattle - Human (Band *et al.* 2000). The interest in comparative mapping of non-mammalian species is increasing, e.g. zebrafish (Woods *et al.* 2000) and salamander (Voss *et al.* 2001). For chicken, a preliminary Chicken - Human comparative map has been made based on linkage data of mapped genes (Groenen *et al.* 2000). There are 300 genes mapped in chicken for which an human orthologue has been identified (Schmid *et al.* 2000). This number of mapped genes with a human orthologue is low; therefore a considerable effort is needed to increase this number and to exploit the human genome sequence data. Nanda *et al.* (1999) showed homology between chicken chromosome Z (GGAZ) and human chromosome 9 (HSA9). Chromosome painting has revealed homology between HSA4 and GGA4 (Chowdhary & Raudsepp 2000). Further, using the Wageningen chicken bacterial artificial chromosome (BAC) library a comparative map between GGA10 and HSA15 has been established (Crooijmans *et al.* 2001).

In chicken, 6 genes whose orthologues are all located on HSA5 (5q23 to 5q35) are mapped on GGA13 (Schmid *et al.* 2000). A much higher gene density, however, is needed to allow a good comparison with HSA5. The aim of this study is to improve the comparative map between GGA13 and HSA5.

MATERIALS AND METHODS

Identification of BACs

The Wageningen chicken BAC library was screened by polymerase chain reaction (PCR) using microsatellite markers known to be located on GGA13 as previously described (Crooijmans *et al*. 2000). Polymerase chain reaction was performed in 5 min at 95 °C and 36 cycles of 30 s 95 °C, 30 s annealingstemperature, 30 s 72 °C, followed by a final elongation step of 10 min at 72 °C. Primers corresponding to genes mapped to GGA13 (*CAMLG*, *CDX1*, *IRF1, MSX2*, *POU4F3, SPARC*) were designed based on database sequences or were obtained from the literature (Table 1) (Schmid *et al.* 2000). BAC-end sequencing of positive

BAC clones was performed as described by Crooijmans *et al*. (2001). BAC-end sequences were used for the development of new STS markers which were subsequently used to screen the chicken BAC library for chromosome walking (http://www.zod.wau.nl/vf/).

Identification of genes

Genes were identified using a bi-directional approach. Subclone sequencing of positive BAC clones was performed as described by Crooijmans *et al*. (2001). Sequences obtained from BAC-ends and BAC subclones were analysed with the PREGAP4 programme of the STADEN software package (Bonfield *et al.* 1995; http://www.mrc-lmb.cam.ac.uk/pubseq). Sequences of good quality reads were used in a BLAST database search (Altschul *et al.* 1997).

Human sequences of genes located on HSA5q23-5q35 were derived from the Unigene database (http://www.ncbi.nih.nlm.gov/UniGene) and subsequently were used to find chicken orthologues performing a BLAST database search (Altschul *et al.* 1997). Chicken sequences (mRNA, expressed sequence tag (EST) or genomic DNA) were used to design gene specific primers (Table 1).

Mapping of genes

Two-colour fluorescent *in situ* hybridisation (FISH) of positive BAC clones was performed as described by Trask *et al*. (1991) and Crooijmans *et al.* (2001). The BAC clones used to identify GGA13 were: bW125K22 from marker *MCW0104* and bW014G12 from marker *MCW0244*. BAC clones isolated for four different genes were used for FISH.

Mapping of an STS marker derived from a BAC clone positive for the gene of interest was performed by single nucleotide polymorphism (SNP) typing. The SNP was first detected in the parents of the East Lancing (EL) (Crittenden *et al.* 1993) and the Wageningen mapping population (WAU) (Groenen *et al*. 1998). The SNP was mapped either on the EL population (52 animals) or on one selected family from the WAU mapping population (42 animals). Before sequencing, the STS amplification products were purified on a P-100 Biogel column (Bio-Rad Laboratories, Hercules, CA, USA).

TABLE 1 STS-markers in genes.

Gene	BAC clone	Accession no. (chicken)	Accession no. (human)[1]	Primer sequences (5'-3')	Primer sequences (5'-3')	Size (bp)
CAMLG	bW025H18	U44473		CCGGTCTGTATGCCTTTAAC	ATGACTGGATGTAAGGCTCC	95
CDX1[2]	bW008N24	U80614		TCTGCCATCATTCTGGCAGG	GCTGTCGGCCACCTGTTGTG	335
IRF1	bW013D15	L39766		AGACCTGAAGGGAATGGCAG	ATTGGTGCCCTGTGTGTGAG	144
MSX2	bW027P20	U14604		AGGAGGTTTTCTCCTCCGAC	TTCTTGTCGGACATGAGGGC	112
POU4F3	bW007A19	X91997		CAACATGGTGGCCCTCAAAC	TGTCACCTCCCGTGTAGATC	104
SPARC	bW001N22	L24906		GACTTCGACAAGTACATCGC	CCAGATCCTTGTCTATGTCC	79
DTR[3]	bW009A03	AF131224	NM_001945	TGTCATCATCACAGCCTTGC	GCATCCTCAGTGATTCACAG	109
GABRA1[3]	bW015N11	X54244	NM_000806	GTTTTGTCTTTTGCTCTGGG T(26)ACAGAACAGAAGTATGT TTCTAACTG[3]	TCTCTCCCTTGTATATAGGG	137
GABRA6[3]	bW010E11	X94343	S81944	GCAGATCAGTCTCCTGAAGC	CTTGTGCCTCCAACAGGTGG	110
GABRG2[3]	bW012D24	X54944	X15376	AAGCAGAAGGAGATCAGTGA T(36)CAAAGAAGCAAGAAGTC ACTCTAA[3]	TCAGACCACTATAAAGCGCG	107
NR3C1[3]	bW046D06	AY029202	NM_000176	AGCTTGGAAAAGCCATCGTG	TTTCTGCCAACATTTCTGGG	180
MADH5[3]	bW099B23	AF143240	NM_005903	AGCTCAGTCTGTCAACCATG	TGCTTGTGACATCTTGTCGG	119

Primers made are based on the chicken genes (accession no.chicken).

[1] Human accession numbers used in a BLAST database search to identify chicken orthologues genes.

[2] Primer sequences are from Kathib *et al.* 1995.

[3] Genes mapped in this paper.

[4] Primer (forward) used in the SNaPshot method.

The quantities of the purified amplification products were measured on a 1.5% EtBr stained agarose gel with a SmartLadder (Eurogentec, Seraing, Belgium). Sequence reactions of the amplification products (100 ng) were performed with the forward or the reverse PCR primer (0.8 pmole/µl). Before analysis of the sequences, sequence reactions were purified on Sephadex G-50 Superfine columns (Amersham Pharmacia Biotech, Dübendorf, Switzerland) in MAHVN 4510 plates (Millipore, Molsheim, France) according to the Millipore protocol.

Sequencing conditions and sequence analysis were performed as described by Crooijmans *et al.* (2001). All sequences obtained were analysed with the PREGAP4 programme of the STADEN software package before analysis with the GAP4 programme to identify SNPs and if possible restriction enzymes specific for the SNPs (Bonfield *et al.* 1995; http://www.mrc-lmb.cam.ac.uk/pubseq). When a specific restriction enzyme was found, the gene was mapped as a PCR-RFLP. In those cases where no restriction enzyme was found, the gene was mapped by single base extension using the SNaPshot -protocol (Applied Biosystems, Inc., Foster City, CA, USA) on an automated seqeuencer ABI377 (Applied Biosystems).

RESULTS

Development of GGA13 BAC contigs

In total, 15 microsatellite markers known to be located on GGA13 were used as starting points for building BAC contigs. In addition, STS markers for 6 genes mapped to GGA13 were used to increase the number of starting points (Table 1). BAC-ends were sequenced and 57 STS (Accession no.: BH126251, BH405309 - BH405364) markers were developed for chromosome walking (http://www.zod.wau.nl/vf/). Eventually, the screening resulted in 204 BAC clones divided over 20 contigs on GGA13. On average 4.3 BAC clones per marker were obtained. Only one marker (*ST13BE053*) did not give any new BAC clones. Interestingly, this marker is located on the contig of *MCW0104*, this marker is located as the end of GGA13 (Figure 1). Three out of 20 BAC contigs were made by linking: the BAC contig around anchor loci *LEI0251* and *ADL0310*, with a genetic distance of 4 cM, the BAC contig around anchor locus *MCW0322* and *CAMLG* and finally the BAC contigs of *ADL0214* and *DTR* (Figure 1).

Identification and mapping of genes

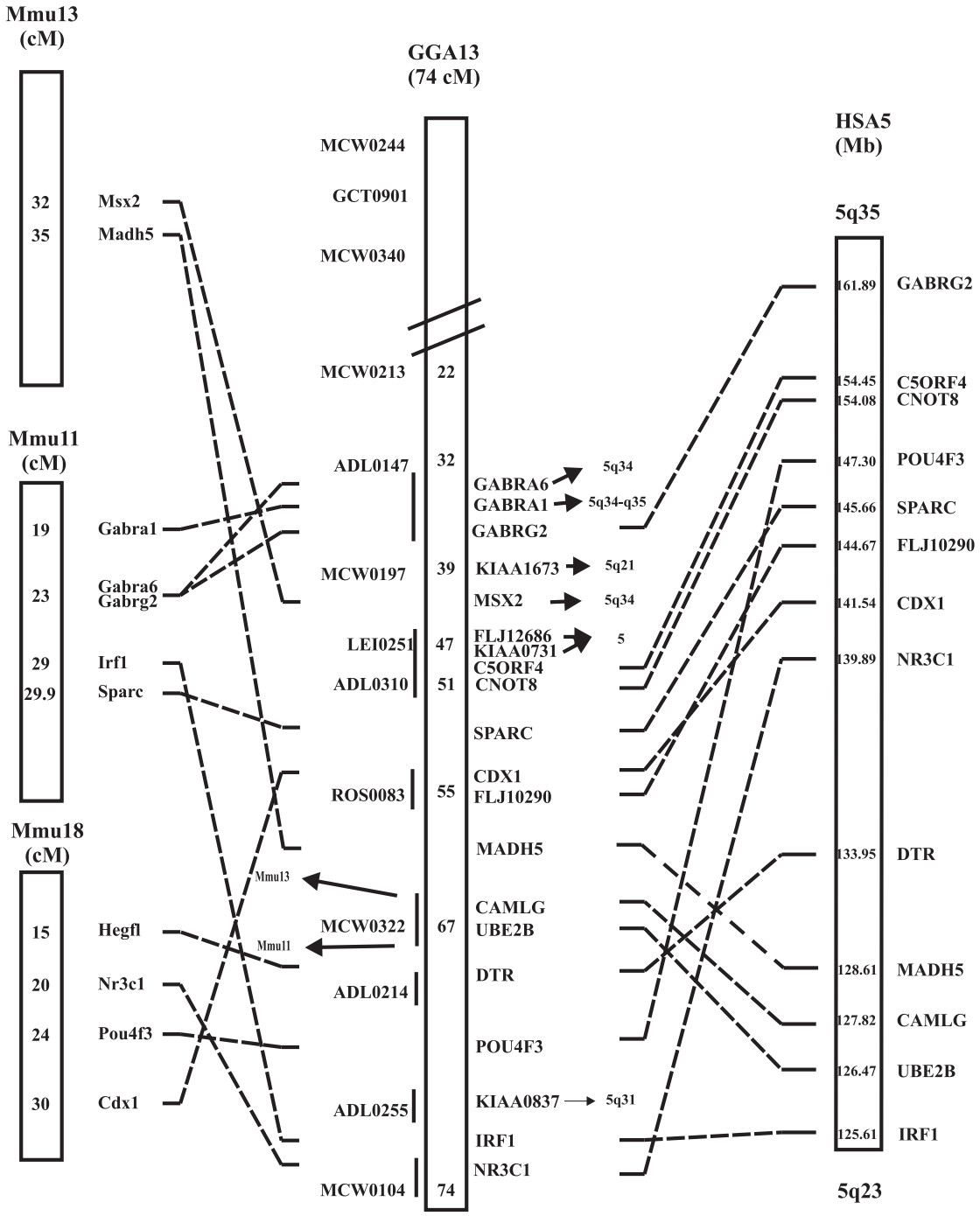
To increase the number of genes on GGA13, 39 BAC clones from 20 GGA13 contigs, were selected for subcloning and sequencing. These BAC clones are equally distributed over the 20 contigs. For each BAC clone 12 randomly picked subclones were sequenced both forwards and reverse. Sequences from the subclones were used in a BLAST database search. In total, eight genes with a human orthologue, two chicken ESTs and one *Columbia livia* EST were identified by sequence comparison (Table 2).

TABLE 2. Genes identified by BLAST comparison of BAC sample sequences.

Locus[1]	BAC clone	Accession no.[2]	Hit	Accession no. hit	bp homology	% identity	Cytogenetic map position
ST13BE22	bW008H08	BH126811	**Chicken EST**	AF095939	73	94	
MCW0340	bW036D13	BH126810	**Columbia livia EST**	AB01792	71	84	
MCW0197	bW123N13	BH126248	KIAA1673	AB051460	380	88	5q21
ST13BE001	bW055A07	BH126250	FLJ12686	AK022748	66	86	5
ADL0310	bW057F10	BH126243	KIAA0731	AB018274	163	83	5
ADL0310	bW010C22	BH126244	C5ORF4	AF159165	142	75	5q31-q32
ADL0310	bW010C22	BH126245	CNOT8	NM_004779	155	86	5q31-q33
ST13BE030	bW008N24	BH126812	**Chicken EST**	AJ395563	494	99	
ROS0083	bW006H13	BH126251	FLJ10290	NM_018047	131	83	5p14.2-q31.3
MCW0322	bW028F16	BH126246	UBE2B	NM_003337	88	86	5q23-q31
ADL0225	bW032D22	BH126249	KIAA0837 (FACL6)	AB020644	125	85	5q31

[1] The most likely locus order according the chicken linkage map (Schmid *et al.* 2000).
[2] Accession number of BAC sample sequences.
BLAST hits of chicken ESTs and columbia livia EST are in bold.

FIGURE 1 Comparative map between mouse-chicken-human. Mouse chromosomes (Mmu) 11, 13 and 18 are indicated as vertical bars on the left hand side (not on scale). The genetic distance is indicated in centiMorgan (cM). Mouse genes are right from the chromosomes. Chicken chromosome 13 (GGA13) is in the middle (not on scale). The genetic distance is in centiMorgan (cM), on the left-hand the chicken loci are indicated according Schmid *et al.* (2000). BAC contig around a marker where a gene was found is indicated as a vertical bold bar. Genes are indicated right from the chromosome. Human chromosome 5 (HSA5) is indicated as a vertical bar on the right hand side in a sequence map location of 160 -125 Mb (not on scale). The gene order is according Map Viewer Build 22 (http://www.ncbi.nlm.nih.gov/) indicated in Megabases (Mb). Genes are indicated right from the bar.



Six chicken genes were identified by the BLAST database search using human sequences specific for HSA5 were used for STS marker development to identify new BAC clones (Table 1). For every gene, one selected BAC clone was used for two-colour FISH and was end sequenced to develop new STS markers. Four of these 6 genes (*DTR*, *GABRG2, GABRA1* and *MADH5*) were mapped to GGA13 using two-colour FISH as described by Crooijmans *et al.* 2001 (data not shown). STS markers derived from BAC end sequences were used for SNP detection. Using PCR-RFLP, two genes (*GABRA6*, *MADH5*) could be mapped genetically on GGA13. Restriction enzyme *Bsr*SI was used to map the BAC clone of locus *GABRA6* on the EL linkage map at 35.8 cM, between *ADL0017* (16.7 cM) and *ADL0147* (44.9 cM). Restricion enzyme *Sau*3A was used to map the BAC clone of locus *MADH5* on the WAU linkage map. *MADH5* was mapped close to *MCW0110* (recombination fraction = 0; LOD score = 12.34). The SNaPshot method was used to map *GABRA1* and *GABRG2* genetically to GGA13 (Table 1). BAC-end STS marker, (forward 5'-AGGGTGTTGCCTTCTAAAGC-3', reverse 5'-CTGGGATATGTGGAATGCTG-3') for BAC clone bW015N11 positive for *GABRA1* is 374 bp in length with an SNP (C ↔ T) on position 255, was mapped on the WAU mapping population. *GABRA1* was mapped close to *MCW0197* (recombination fraction = 0.10; LOD score = 6.35). BAC-end STS marker, (forward 5'-TGAATCCAGAGCACTCTCTG-3', reverse 5'-GAAGCACAGGCTAGTTTTGC-3') for BAC clone bW012D24 positive for *GABRG2* is 357 bp in length with an SNP (C ↔ T) on position 258, was mapped on the EL mapping population at 38.1 cM between *ADL0017* (16.7 cM) and *ADL0147* (44.9 cM). Subsequently, BAC-ends of BACs positive for *GABRA6*, *GABRA1* and *GABRG2* were sequenced and STS markers were developed for chromosome walking. The screening resulted in a contig containing *GABRA6*, *GABRA1* and *GABRG2*. One gene (*NR3C1*) was directly mapped to BAC clones that had already been identified by loci located on GGA13. The total number of genes assigned to GGA13 increased to 20 (Figure 1). For all genes mapped to GGA13 up to now an orthologue in human on HSA5q23-5q35 has been identified, except for *KIAA1673* which has a cytogenetic map position of HSA5q21. In mouse, the orthologues map to three different chromosomes Mmu11, Mmu13 and Mmu18.

DISCUSSION

We have used the detailed mapping information of human and mouse to identify genes that map to chicken chromosome 13 (GGA13) by using a bi-directional approach. The size of GGA13 is about 74 cM in length, which is about 2% of the total chicken genome (3800 cM; 1200 Mb) (Groenen *et al.* 2000). Identification of 204 BAC clones (average insert size 130 kb) on GGA13 has resulted in an estimated coverage of about 20% of this chromosome, based on the Wageningen chicken BAC library containing 50 000 clones with a 5.5 times coverage of the chicken genome (Crooijmans *et al.* 2000). The number of BAC clones per marker in our study range from 1 to 11 with an average of 4.3, which is somewhat lower than the estimated 5.5 times coverage of the chicken genome (Crooijmans *et al.* 2000).

Eight genes found by sample sequencing of chicken BAC clones all correspond to human orthologues located on HSA5 (Table 2). Two sequences have a relatively small region of sequence identity (BH126250, BH126246) to genes in human; however, the sequence identity is over 80%. For BH126250 the sequence identity is in the coding region of *FACL6* (*KIAA0837*) and is, therefore, considered to be the orthologue in chicken. For BH126246, the homology is in the non coding region in the 3' end of the *UBE2B* gene. Moreover, sequence comparison of a subclone of an overlapping BAC clone (bW006N15) within the same BAC contig also showed sequence identity to the 3' end of the *UBE2B* gene (data not shown). Therefore, it is suggested that the chicken *UBE2B* gene is within this contig. The sequences used in this study are from both coding and non-coding regions showing homology to human sequences. This indicates that even after 300 million years of evolution potential regulatory elements can be identified using this method. One orthologous gene has a cytogenetic map position of HSA5q21, which is outside the range HSA5q23-5q35. It is possible that the comparison of GGA13 and HSA5 should be extended to the 5q21 region; however, mapping genes based on their cytogenetic map position is not very accurate. Therefore, it is likely that *KIAA1673* still fits in the range based on the sequence map position (125 - 162 Mb). The advantage of identifying chicken orthologues by BAC sample sequencing is that the gene is directly mapped on the chicken linkage map. The efficiency of identifying genes by BAC sample sequencing, however, is low. Therefore, a bi-directional approach was used. In total, 12 chicken genes with high sequence identity to human genes on HSA5q23-5q35 were identified by BLAST database searches. Five of these chicken genes were mapped on GGA13 either by FISH and/or SNP detection (Table 1). Two genes could not be mapped either

because no SNP could be found within the STS fragment [*Hsp70kd* (Accession no.: J02579)] or because no BAC clone could be identified [*FGF1* (Accession no.: S63263)]. In our study, 2 chicken genes [*CTNN* (Accession no.: D11090) and *Phosphatase 2A* (Accession no.: D17531)] with high sequence identity to human genes located on HSA5 were mapped to chicken chromosomes other than GGA13. These genes were part of a gene family, e.g. chicken *CTNN* (Accession no.: D11090) maps to GGA4 instead of GGA13 (data not shown). The human gene *CTNNA1* (Accession no.: NM_001903) on HSA5 has high sequence similarity to the chicken *CTNN*. However, *CTNN* has also high sequence identity to human *CTNNA2* (Accession no.: XM_010873) located on HSA2p12-p11.1. The chicken *CTNN* has been mapped on GGA4 close to (2.2 cM) *CD8A*, which is located in human on HSA2p12. Therefore, it is likely that the chicken *CTNN* is not the orthologue of human *CTNNA1*, but the orthologue of human *CTNNA2*.

Chicken *Phosphatase 2A* (Accession no.: D17531) has a high sequence identity with the human gene *PPP2CA* (Accession no.: XM_057748) on HSA5q23-q31 as well as the human *PPP2CB* (Accession no.: XM_053511) on HSA8p12-p11.2. In chicken *Phosphatase 2A* (Accession no.: D17531) maps to GGA4; therefore, it is likely that *Phosphatase 2A* is the ortholog of human *PPP2CB*. Chicken genes found with high sequence similarity to human genes can be the paralogous instead of the orthologous gene. This is a disadvantage of using human sequences to find chicken genes, because in human there are larger gene families (Burt *et al.*1999). The examples mentioned above show that it is important to use the combination of both data types of sequence homology as well as conserved linkage to establish orthologies within gene families. We have improved the comparative chicken - human - mouse map by mapping an additional 14 genes on GGA13, thus thereby increasing the total number of genes mapped to this micro-chromosome to 20 (Figure 1).

During evolution in the lineages leading to chicken, mouse and human, more inter chromosomal rearrangements in the mouse genome can be found than in the chicken genome in comparison to the human genome (Burt *et al.* 1999). Genes mapped to GGA13 have their orthologues genes on 3 different mouse chromosomes (Mmu11, Mmu13, Mmu18) and one human chromosome (HSA5). This is in accordance with results of Chowdhary & Raudsepp (1999) and Crooijmans *et al*. (2001), who have also shown more inter chromosomal rearrangements between chicken - mouse than chicken - human for GGA4 and GGA10, respectively. Conserved chromosomal segments can be distinguished in chicken (Burt *et al.* 1999; Nanda *et al.* 1999; Groenen *et al.* 2000); however, many intra-chromosomal

rearrangements in chicken were observed (Crooijmans *et al.* 2001). There is a conserved region comparing GGA13 to HSA5q23-5q35. An intra-chromosomal rearrangement is likely between *CDX1* and *NR3C1*. These genes are close together within a 2 Mb interval in human. However, in chicken these genes are much more separated (19 cM apart) (Figure 1). To identify the actual breakpoint more accurately, additional genes need to be mapped around *CDX1* and *NR3C1*.

In conclusion, we have improved the comparative map by mapping 14 additional genes and 57 STS markers to GGA13. The chicken-human-mouse map shows homology between GGA13-HSA5q23-5q35 - Mmu11, Mmu13, Mmu18.

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CHAPTER 5

Identification of Quantitative Trait Loci for Receiving Pecks in Young and Adult Laying Hens

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ABSTRACT

Feather pecking (FP) is a major problem in both cage and free range housing systems. In free range systems, FP is more difficult to control. It is not known why a victim is being pecked. It could be that a bird is genetically predisposed to be pecked. To study the genetics of FP behavior, a large F_2 population of 630 hens was generated from a cross between two commercial laying lines differing in their propensity to feather peck. The traits measured at 6 and 30 wk of age were: receiving gentle FP, receiving severe FP, and receiving aggressive pecking. In addition, receiving toe pecking (TP) was also measured at 30 wk of age. For receiving gentle FP at 6 wk of age, a significant QTL on GGA1 and three different suggestive QTL were identified on GGA2, GGA6, and GGA7, respectively. For receiving gentle FP at 30 wk of age, a suggestive QTL on GGA5 was detected. For receiving aggressive pecking, a suggestive QTL was detected on GGA3. For receiving TP, three suggestive QTL were detected on GGA1, GGA5, and GGA23, respectively. The different QTL detected for receiving gentle FP at different ages indicate that this trait is regulated by different genes in young and adult hens.

(*Key words:* behavior, chicken, feather pecking, quantitative trait locus, receiving pecks)

INTRODUCTION

Feather pecking (FP) is a major problem in both cage and free range housing systems. In free range systems FP is more difficult to control. Legislation concerning animal welfare is becoming more strict in the European Union (Blokhuis et al., 2000). This legislation results in more free range housing systems. Beaktrimming is an effective method to reduce feather damage due to pecking behavior (Blokhuis and Wiepkema, 1998). However, this method is not an animal friendly method because it can cause pain and neuromas (Gentle, 1986, 1991). Unfortunately, animal friendly alternative methods are not available at the present time.

Five different forms of (bird - to - bird) pecking were described (Savory,1995): aggressive pecking, gentle FP, severe FP, tissue pecking, and vent pecking. Aggressive pecking is the pecking aimed at the head of the subordinate and can cause severe damage to the comb. Receiving birds may vocalize and withdraw. Gentle FP is the pecking that causes little or no damage to the bird. It appears that it is directed at particles on the feathers of the receiving bird. In adult chickens, gentle FP can develop into stereotyped gentle FP. Receiving birds seem unharmed. Severe FP is one that causes feather damage and loss. Severe FP is painful for receiving birds. The receiving bird may react differently. Some may vocalize and withdraw, and others may also react with an aggressive peck to peckers. Tissue pecking in denuded areas are forceful and aimed at a denuded spot. Receiver avoid the pecking by moving away. Eventually, it may give up and submits to the pecking. Vent pecking is aimed at the cloaca. It may start as an investigatory pecking and can result in cannibalism (Savory, 1995). A sixth form of pecking is toe pecking (TP). The TP is pecking aimed at the toes of the conspecific. Receivers react and withdraw.

Most studies on FP behavior focus on the peckers, however, one can also ask the question why a bird is pecked. One explanation could be that the plumage of one bird is more interesting to peck at than the other bird. It has been shown that manipulation of the feathers induced more FP (McAdie and Keeling, 2000). Another explanation could be that the social hierarchy is playing a role. However, the lack of social structure in large flocks may be a factor of minimizing pecking between individuals (Hughes et al., 1997). Therefore, it is of interest to find out if a bird is genetically predisposed to be the receiving bird for different forms of pecking. Kjaer and Sørensen (1997) have shown that receiving pecking is heritable in young chicken; however, in adult chicken the heritability did not differ significantly from 0. However, Rodenburg et al. (2003) did not find a heritability differing significantly from 0,

either in young chicks or in adult hens. It was suggested that receiving FP is more influenced more by the social structure of groups than by pecking trait itself (Kjaer and Sørensen, 1997).

The progress made in the development of a genetic linkage map (Groenen et al., 2000) and the development of molecular tools such as the chicken Bacterial Artificial Chromosome library (Crooijmans et al., 2000) enables the dissection of the molecular basis of complex quantitative traits such as behavior. To date, not many QTL studies of chicken behavior have been performed. Only recently, QTL studies indicated that it is feasible to detect QTL for social tendency and contrafreeloading behavior (Schütz et al., 2002) as well as for FP behavior (Buitenhuis et al., 2003), and open-field behavior (A. J. Buitenhuis, unpublished data). In the search for a viable way to improve animal welfare in poultry, insight in the genetic architecture of receiving FP might be useful in comparing the peckers with the receivers. The aim of the present study is to identify QTL involved in receiving FP behavior at 6 and at 30 wk of age in an F_2 population from a cross between a high FP (HFP) and a low FP (LFP) line.

MATERIALS AND METHODS

Experimental Population

A cross between two commercial laying lines was made to produce 630 F_2 hens as previously described (Rodenburg et al., 2003; Buitenhuis et al., 2003).These lines differ consistently for behavioral traits such as FP and open-field behavior (Blokhuis and Beutler, 1992; Jones et al., 1995) as well as for physiological traits (Korte et al., 1997; Van Hierden et al., 2002). The F_2 hens arrived at the experimental farm as day-old-chicks in 5 batches in 2-wk intervals. The birds were not beak-trimmed and each individual bird was marked with a wing-band. Each batch was divided over 2 pens, giving a total of 10 groups (batch x pen) with an average of 63 birds per group. The floor area of the pen was 4.75 x 2 m and covered with wood-shavings. Each pen had 2 light tubes (2 x 40W) and during wk 0 to 4 a heating lamp was available. From wk 0 to 4 there was continuous light from the heating lamp, while in wk 5 to 6 the scheme was changed to 8 h light per day from 8:00 to 16:00 h. From 16 wk of age onwards the light scheme was extended 1 h/wk. The animals had 16-h light day from 0300 to 1900 h at 24 wk of age. Feed (152 g/kg crude protein and 2,817 kcal/kg metabolizable energy) and water was provided for ad libitum consumption (Rodenburg *et al.*, 2003; Buitenhuis *et al.*, 2003).

Phenotypic Data

A social FP test (30 min) was performed at 6 wk of age on 625 F_2 birds and at 30 wk of age on 550 F_2 birds. The social FP test, as used in the current study, was described in detail by Rodenburg et al. (2003). In brief, five birds from the same home pen and batch were randomly assigned to the test group and transferred to a sound attenuated room. The social FP test was performed in an observation pen of 1.25 x 1.25 m with wood shavings on the floor. The birds were placed in the observation pen in darkness. The observation started when two light tubes of 40 W each, were switched on. Behavior of the hens was recorded directly from an adjacent room using a video camera.

The traits measured at 6 and 30 wk of age were: 1) receiving gentle FP (gentle peck, no reaction of the receiving bird); 2) receiving severe FP (severe peck, reaction of the receiving bird); and 3) receiving aggressive pecking (dominance peck aimed at the head, neck, or back, reaction of the receiving bird). Moreover, receiving TP (severe peck aimed at the toe or leg, reaction of the receiving bird) was also measured at 30 wk of age. A detailed description concerning the distribution, averages, and standard deviation of the traits is given (Rodenburg et al., 2003), except for toe pecking. The Wageningen University Committee on Animal Care and Use, The Netherlands, has approved the use of the birds in the current experiment.

Genotypic Data

Genotyping of the F_0, F_1, and F_2 generation, has been previously described in detail (Buitenhuis et al., 2003). From 5 wk-old-birds blood was taken from the wing veine. The DNA was extracted according the Capture Plate^{TM} Kit protocol[1]. The F_0, F_1, and F_2 generation were genotyped with a total of 180 micro-satellite markers, covering GGA1-GGA19, GGA23, GGA24, GGA27, GGA28, and GGAZ and linkage groups E38, E47W24, and E60E04W23 (Groenen et al., 2000). Markers were labeled with a fluorescent dye (6-FAM, HEX, or TET). The amplification reaction was performed as described by Crooijmans et al. (1997) with slight modifications. The PCR program consisted of 5 min denaturation at 95°C, 36 cycles of 30 s at 95°C, 30 s at annealing temperature, and 30 s at 72°C followed by a final elongation step of 10 min at 72°C. Fragment sizes were calculated relative to the GENESCAN-350 TAMRA

[1] Gentra Systems, Minneapolis, MN, 55447

marker[2] with GENESCAN 2.1 fragment analysis software[2] and allele identification was performed using GENOTYPER 2.0 software[2]. All genotypic data were checked by two independent individuals prior to inheritance checking using CRI-MAP (Green et al., 1990).

Genome-Wide Scan

Before the total genome scan analysis, data was adjusted using the PROC GLM procedure (SAS Institute, 1985) with testgroup (j = 1,2,....,129) (group in which the birds were tested) as fixed effect in the model at 6 wk of age. The data at 30 wk of age were also adjusted with testgroup (j = 1,2,….,112) as a fixed effect in the model. The effects of home pen (pen in which the bird is housed) and batch (order in which the birds arrived at the farm) are accounted for by testgroup [pen (batch)].

A regression method was used for interval mapping. Two different genetic models were used: a paternal half-sib analysis (Knott et al., 1996; De Koning et al., 1999) and a line-cross analysis (Haley et al., 1994; De Koning et al., 2000). In the paternal half-sib model no assumptions were made concerning the allele frequencies in the founder lines and the number of QTL alleles. The F_2 animals are treated as 7 unrelated half-sib families using the model:

$$Y_{ij} = m_i + b_i p_{ij} + e_{ij}$$

where Y_{ij} = trait measured on animal j, from rooster i; m_i = average of half-sib family i; b_i = substitution effect for a putative QTL; p_{ij} = the conditional probability for animal j of rooster i inheriting the first paternal haplotype; and e_{ij} = residual effect. In this analysis the contrast between the two haplotypes of every F_1 rooster was made. Analyses within families were performed and the test statistics was calculated as an F ratio for every centi Morgan along the genome (De Koning et al., 1999).

In the line-cross model (Haley et al., 1994), the alternative alleles at the QTL were traced back to the founder lines. De Koning et al. (2000) have adapted the model for the detection of parent-of-origin effects containing a paternal, a maternal, and a dominance component:

$$Y_j = m + a_{pat} p_{patj} + a_{mat} p_{matj} + d p_{dj} + e_j$$

[2] Applied Biosystems, Perkin Elmer, Foster City, CA 94404

where Y_j = trait measured on animal j; m = population mean; a_{pat}, a_{mat} = additive effect; d = dominance effect; p_{pat} = conditional probability that an animal inherited a HFP allele from the sire ($[p_{11} + p_{12}] - [p_{22} + p_{21}]$); and p_{mat} = conditional probability that an animal inherited a HFP allele from the dam ($[p_{11} + p_{21}] - [p_{22} + p_{12}]$).

The threshold levels were empirically determined using a permutation test for each chromosome (Churchill and Doerge, 1994). The threshold was determined using 10,000 permutations. The threshold levels as suggested by Lander and Kruglyak (1995) were used: 1) suggestive linkage (statistical evidence expected to occur one time at random in a genome scan); and 2) significant linkage (statistical evidence expected to occur 0.05 times in a genome scan), i.e., a 5% genome-wide threshold.

For QTL with possible parent-of-origin effect an additional test of the full model against the mendelian model was performed. The genome was screened for imprinted QTL using an imprinted model (either maternally or paternally). At locations where significant evidence for the presence of an imprinted QTL was found, it was tested if an imprinted QTL explained the observations better than a mendelian model (Knott et al., 1998). The test used was an F test with 1 d.f. in the numerator and (n-4) d.f. in the denominator. The test of Knott et al. (1998) is in general somewhat more conservative than the test proposed by De Koning et al. (2000) as reported in De Koning et al. (2002).

RESULTS

Half-Sib Analysis

The results of the half-sib analysis of the behavior traits are presented in Table 1. For receiving gentle FP at 6 wk of age, three different QTL were detected. 1) A genome wide significant QTL was detected on GGA1 between marker brackets ROS0081 and MCW0273 (Figure 1). Out of the seven half-sib families, one family (family 4) contributed most to the overall QTL with an allele substitution effect of –1.69 and an F ratio of 14.57; 2) A suggestive QTL on GGA6 was detected between marker brackets HUJ0005 and MCW0325. One family (family 4) contributed most to the overall QTL with an allele substitution effect of –1.13 and an F ratio of 11.27; and 3) A suggestive QTL was detected on GGA7 between marker brackets MCW0236 and LEI0158. For this QTL, one family (family 6) contributed most to the QTL with an allele substitution effect of 0.81 and an F ratio of 7.87. For both receiving severe FP and receiving aggressive pecking at 6 wk of age, no QTL were detected.

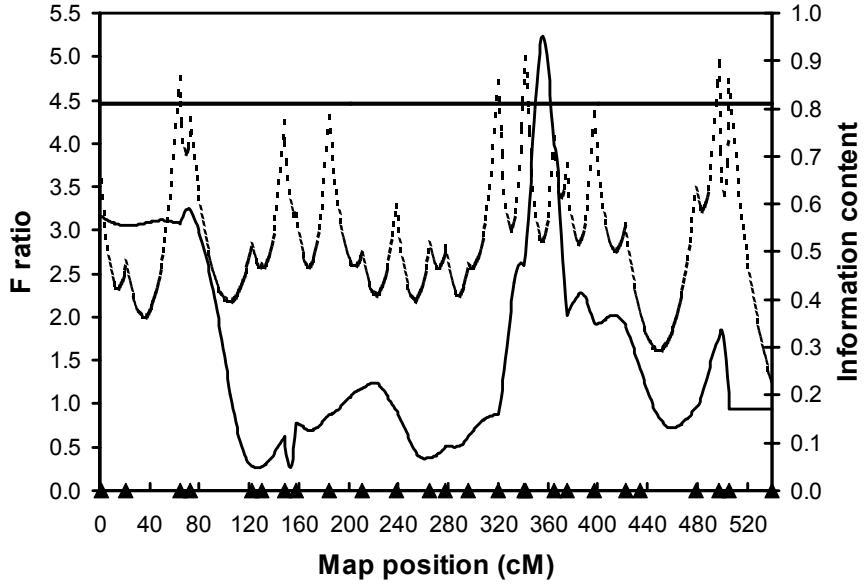
TABLE 1. QTL affecting receiving pecking behavior using the half-sib analysis.

Trait	Chromosome	Position (cM)	Marker bracket	F[1]
		6 wk of age		
Receiving gentle FP[2]	GGA1	356	ROS0081-MCW0273	5.23*
	GGA6	4	HUJ0005-MCW0325	3.50†
	GGA7	114	MCW0236-LEI0158	2.72†
		30 wk of age		
Receiving gentle FP[2]	GGA5	166	MCW0029-ADL0298	3.11†

[1]F ratio: †suggestive linkage; *genome wide significance at 5% level.
[2]FP = feather pecking.

For receiving gentle FP at 30 wk of age, only one suggestive QTL on GGA5 was detected (Table 1). Two half-sib families out of the seven contributed to this QTL. One family (family 2) had an allele substitution effect of –0.91 and an F ratio of 11.34, and the other family (family 4) had an allele substitution effect of –0.92 and an F ratio of 10.20. For receiving severe pecking, receiving aggressive pecking, and receiving TP at 30 wk of age, no QTL were detected.

FIGURE 1. QTL for receiving gentle feather pecking at 6 wk of age on GGA1 under the half-sib analysis. The F-statistics profile is given as a solid line. The information content is given as a dashed line. The horizontal line refers to the 5 % genome wide significance level. Marker positions on the chromosome are indicated as triangles.



Line-Cross Analysis

The results of the line-cross analysis of the behavior traits are presented in Table 2. For receiving gentle FP at 6 wk of age, a suggestive QTL was detected on GGA2 between marker brackets MCW0087 and ROS0023. For receiving severe FP and receiving aggressive pecking, no QTL were detected at 6 wk of age.

For receiving aggressive pecking at 30 wk of age, a suggestive QTL was detected on GGA3 between marker brackets MCW0252 and MCW0006. For receiving TP three QTL were detected: 1) on GGA1, a QTL was detected almost reaching the genome wide significance threshold (P = 0.06) between marker brackets MCW0109 and MCW0046; 2) a suggestive QTL was detected on GGA5 between marker brackets ADL0023 and MCW0029; and 3) a suggestive QTL was detected on GGA23 between marker brackets ADL0289 and LEI0090. The average of number (SD) of TP was 0.13 (1.09). It occurred in 3% of the animals in this population, and was present in only 2 out of the 10 home pens. For the traits receiving gentle FP and receiving severe FP at 30 wk of age, no QTL were detected. When testing for parent-of-origin effect, no QTL were detected for any traits at 6 or 30 wk of age.

TABLE 2. QTL affecting receiving pecking behavior using the line-cross analysis.

Trait	Chromosome	a (SE)[1]	d (SE)[1]	Position (cM)	Marker bracket	F[2]
		6 wk of age				
Receiving gentle FP[3]	GGA2	-0.7 (0.2)	-1.4 (0.5)	269	MCW0087-ROS0023	7.58[†]
		30 wk of age				
Receiving aggressive pecking	GGA3	-5.9 (0.9)	-17.1 (2.8)	231	MCW0252-MCW0006	21.85[†]
Receiving toe pecking	GGA1	4.7 (1.6)	-35.1 (5.5)	314	MCW0109-MCW0046	21.02[†]
	GGA5	4.1 (1.7)	-9.8 (3.1)	77	ADL0023-MCW0029	12.38[†]
	GGA23	-4.9 (1.1)	-6.1 (2.2)	8	ADL0289-LEI0090	14.24[†]

[1]Estimated QTL effects for the genetic model: a = the additive effect; and d = the dominance level. Standard errors are in parenthesis.
[2]F ratio: [†]suggestive linkage; [*]genome wide significance at 5% level.
[3]FP = feather pecking

DISCUSSION

Feather pecking causes economic losses for the farmer and impairs animal welfare (Blokhuis et al., 2000). Recently, QTL were detected for performing FP behavior in chickens (Buitenhuis et al., 2003). However, it would also be of interest to study the genetics from the perspective of receiving birds. Therefore, the aim of the current study was to identify QTL for receiving pecking behavior in young and adult laying hens.

The reason why a bird is pecked could have different causes. In the ranking positions of a group there are birds which are sub-dominant. These birds receive pecks from the dominant group-mate. This type of pecking is referred to as aggressive pecking. The response of the receiving bird is, depending on the social status, active, i.e., it will fight, or passive, i.e., it will try to escape. However, not all pecking is directly related to maintaining the social status of the bird. In the different types of pecking defined (Savory, 1995), motivational differences between the type of FP exist. In general, receiving birds reacted different to the pecks received. In case of receiving severe feather pecks the receiving bird reacted aggressively, i.e., pecked back, froze, or tried to escape. In case of receiving gentle pecks, there was not a clear response of the receiving bird. In case of receiving TP, the receiving bird tried to escape or froze. The TP frequently caused severe wounds at the toes. An explanation why a bird is pecked might be found in the reaction of a receiving bird to the pecks received, e.g., birds which fight back might not be attractive to peck at. Another explanation could be that the feathers were damaged or that blood was exposed to the conspecific.

For the detection of QTL, a paternal half-sib model (Knot et al., 1996; De Koning et al., 1999) and a line-cross model (Haley et al., 1994; De Koning et al., 2000) was used. Because the HFP and LFP lines were not selected for the traits observed in the present study, one did not know what to expect concerning the frequency of favorable alleles. The use of different QTL detection methods could take into account the possible differences in allele frequencies.

For receiving gentle FP, the line-cross analysis revealed QTL different from the ones identified under the half-sib analysis. There was a clear difference between the QTL detected for receiving gentle FP at young and QTL detected at adult age. Both the number and the magnitude of the QTL was higher at the young age than at adult age. This is in agreement with the study of Kjaer and Sørensen, (1997). The heritability for receiving gentle FP at a young age was 0.15, whereas in adult chickens, the heritability was not signifcantly different

from 0 (Kjaer and Sørensen, 1997), indicating that the environmental influence was increasing with age.

It is possible that the motivation of pecking behavior at other birds at different ages changes. Riedstra and Groothuis (2002) suggested that FP at young age is a form of social exploration. This could offer an alternative explanation for the hypothesis that FP is related to foraging behavior (Blokhuis, 1986) or dustbathing behavior (Vestergaard et al., 1993). The social structure has established when the birds are 5 to 6 wk of age (Guhl, 1958). In the current study, birds in one test group were coming from the same pen. Although it is not likely that birds can recognize all of their pen mates (McBride and Foenander, 1962), during the social FP test, aggressive pecking did not occur very often. This indicated that the social structure had already been established. The trait receiving gentle FP at young age may, therefore, be social exploration. There was, however, no direct indication of social interaction between the pecker and receiver. Once a bird in the group started to perform gentle FP, other group mates were attracted to the pecked spot and copied this behavior. This spot could consist of feathers which were damaged and, therefore, these spots were more attractive to peck on than intact feathers (McAdie and Keeling, 2000). If there is no real social interaction needed for receiving gentle FP, being pecked might then be more dependent on the genetic constitution of the pecker then on the genetic constitution of the bird receiving the pecks. Nevertheless, a suggestive QTL on GGA5 was detected for receiving gentle FP in adult laying hens.

For receiving TP, three suggestive QTL were detected using the line-cross analysis, however, no QTL were detected using the half-sib analysis. A reason why the half-sib analysis did not reveal a QTL might be that receiving TP did not occur very often in this population. Only 3% of the birds performed TP. The birds which received TP were randomly distributed over the seven half-sib families. The average number of birds which receive TP within a half-sib family was 2.7. Consequently, the power to detect a QTL was too low in the half-sib analysis. The interpretation of the QTL for receiving TP was not entirely clear. In the social FP test, birds did not show TP when counter birds had intact toes; however, when a bird had an injured toe at the start of the test, the group members focussed on the injured toe. The TP was not shown in all home pens, only in 2 out of the 10 pens birds with injured toes were found. In the pens where the birds had intact toes, no TP was observed. Apparently injured toes, which were likely the result of TP, act as a trigger for (more) TP in the affected pen, because TP did not disappear in the affected pens.

The QTL detected for receiving gentle FP at 6 wk of age were different from those detected at 30 wk of age. This suggested that behavior, as far as receiving pecks can be defined as a type of behavior, at different ages might be regulated by different genes. Therefore, receiving pecks in juvenile chickens should be considered as a different trait than receiving pecks in adult chickens. In addition, when comparing the QTL locations found for receiving gentle FP to QTL detected for performing FP behavior (Buitenhuis et al., 2003), no coinciding regions were found. Based on the current study, it was not possible to exclude the existence of coinciding QTL between different behaviors.

The QTL detected for receiving FP behavior opens the possibility to identify genes underlying behavioral traits in chicken. The essential genetic tools to dissect QTL such as a consensus linkage map (Groenen et al., 2000), and a bacterial artificial chromosome library (Crooijmans et al., 2000) are available. In addition, DNA sequencing of the chicken genome is in progress. The chicken genome sequence will be an indispensible tool to identify candidate genes underlying QTL. Because the chicken diverged from the common ancestor of mammals and birds about 300 Million years ago (Burt et al., 1999), the genetic mechanisms involved in behavior may be different between mammals and birds. Identification of genes underlying different behavioral traits in chicken and comparison to mammals can provide insight to the different aspects of behavior as well as on evolutionary aspects of behavior.

In conclusion, receiving FP at 6 wk of age was regulated by different QTL than receiving FP at 30 wk of age. In general, more QTL for receiving gentle FP in young chickens were detected than in adult chickens. For receiving gentle FP, there were no coinciding QTL detected with the traits performing gentle FP and severe FP, indicating that different behaviors were regulated by different genes.

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CHAPTER 6

Identification of QTL Involved in Open-field Behavior in Young and Adult Laying Hens

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ABSTRACT

Line differences for open-field behavior in chickens have been observed, and it has been shown that this behavior has a genetic component. The aim of this study was to detect QTL involved in open-field behavior. For this purpose open-field behavior was studied at 5 weeks and 29 weeks of age in F_2 hens coming from an intercross between two commercial White Leghorn laying lines selected for egg production traits. Latencies, durations and frequencies of general activity (sitting, standing, walking and stepping), defecation and vocalizations were recorded individually for each bird and a factor score was calculated. All animals (F_0, F_1, and F_2) were screened with 180 micro-satellite markers. Regression interval mapping was applied using a paternal half-sib analysis and a line-cross analysis method. For general activity at 5 weeks of age, a significant QTL was detected on GGA4, and a suggestive QTL on GGA2 under the line-cross model. For general activity at 29 weeks of age, a significant QTL was detected on GGA4 and two suggestive QTL were detected on GGA1 and on GGA10, respectively, also using the line-cross analysis. The QTL on GGA4 at 5 weeks of age did not overlap with the QTL on GGA4 at 29 weeks of age. The current study indicated that open-field behavior in young chicken was regulated by QTL which differ from the QTL for open-field behavior in adult chickens.

(*Key words:* chicken; QTL analysis; open-field; behavior)

INTRODUCTION

Hall (1934) was the first to describe the open-field test in rats. In his study, it was proposed that defecation and urination are valid and consistent measurements to identify individual differences in emotionality (Hall, 1934). Since then many studies have been undertaken to study genetic and environmental effects on emotionality using the open-field test. Results between studies, however, are not always consistent due to variability in testing conditions (Ramos and Mormède, 1998). Several different behavioral measures can be discerned in the open-field test, but apparently there is not one single trait that reflects anxiety or emotional reactivity, at least in mice (Crawley *et al.*, 1997). Genes involved in open-field behavior may be involved in locomotion activity, in exploratory activity as well as in more difficult to define traits like fear or anxiety (Crawley *et al.*, 1997). The recent development of molecular genetics and quantitative trait mapping tools enables the identification of quantitative trait loci (QTL) in the genome involved in complex traits like behavior. To date, most QTL mapping experiments concerning open-field behavior focused on rodents. In rats, Fernandez-Turuel *et al.*, (2002) identified three QTL for activity in the open-field. In mice, Flint *et al.*, (1995) identified six chromosomal regions influencing open-field activity. Three of these QTL accounted for most of the genetic variance. More recently, Turri *et al.*, (2001a,b) identified QTL for open-field activity in mice. Some of these QTL coincide with other ethological tests indicating that these QTL might have pleiotrophic effects.

Behavioral strategies of animals to stimuli in the environment can vary. These coping responses can be classified into distinct coping styles in rodents. Koolhaas *et al*. (1999) defined a coping style as: ‘a coherent set of behavioral and physiological stress responses which is consistent over time and which is characteristic to a certain group of individuals’. The two types of behavioral characteristics could be divided into a proactive and a reactive group. The proactive animals were associated with an active behavioral response (fight/flight), a low corticosteroid level and a high neurosympathetic activity. The reactive animals were associated with a passive behavioral response (conservation/withdrawal), a high corticosteroid level and normal neurosympathetic activity (Koolhaas *et al*., 1999).

Open-field behavior in chickens has been previously compared with open-field behavior in other species (Candland and Nagy, 1969). The behavior of chickens exposed to a fear-inducing stimulus was characterized by freezing and reduced vocalization. Chickens freeze when placed in the center of the field, subsequently followed by an increase of activity

during the trial (much like "reactive" rodents). The behavior of chickens in the open-field generally included an increase of defecation with age, and young animals vocalized more than adults (Candland and Nagy, 1969). Gallup and Suarez (1980) proposed that open-field behavior in chickens could be seen as a combination of opposing reactions to the threat of predators and social separation. Indications for a genetic component for open-field behavior in chickens have been found by different authors (Faure, 1981; Webster and Hurnik, 1989; Rodenburg *et al.*, 2003). The heritability estimates were within the ranges of 0.11 to 0.49 for general activity, and of 0 to 0.22 for defecation. Rodenburg *et al.*, (2003) showed that the heritability estimate of open-field behavior was higher at young age than at adult age. In addition, a selection experiment showed that it is possible to select for open-field behavior in young chicks (Faure, 1981). Selection for production traits could have negative side effects for health in poultry (Rauw *et al.*, 1998). With regard to effects of selection for production traits on behavioral traits; a negative correlation between body weight and feather pecking behavior was found in a female line of White Leghorn layers (Kjaer and Sørensen, 1997). Concerning open-field behavior, line differences have been found between White Leghorn lines selected for production traits (Webster and Hurnik, 1989; Jones *et al.*, 1995).

Tools to dissect the molecular basis of quantitative traits in chicken, such as the chicken linkage map (Schmid *et al.*, 2000), a chicken bacterial artificial chromosome library (Crooijmans *et al.*, 2000) and a chicken radiation hybrid map (Morisson *et al.,* 2002) are available. In addition, sequencing of the chicken genome is in progress. This will greatly benefit the identification of genes involved in the traits of interest. QTL affecting behavior in chickens have been found for social tendency, contrafreeloading (Schütz *et al.*, 2002) and feather pecking behavior (Buitenhuis *et al.*, 2003).

Here we present the first study on QTL mapping for open-field behavior in chickens, in which the genome was scanned for DNA markers associated with open-field behavior, using an F_2 cross originating from two commercial chicken White Leghorn laying lines differing in their open-field response (Jones et al., 1995). This study may be of potential interest in comparative behavioral studies between, e.g., the mouse and chicken, to see if genes playing a role in locomotion traits in mouse, also play a role in locomotion traits in chickens.

MATERIALS AND METHODS

Test Population

The lines used were denoted as high feather pecking line (HFP) and low feather pecking line (LFP). The HFP and LFP lines differ for behavioral traits (Blokhuis and Beutler, 1992; Jones *et al.*, 1995; Rodenburg and Koene, 2003) as well as for physiological traits (Korte *et al.*, 1997; Van Hierden *et al.*, 2002). A reciprocal cross was made of the HFP and LFP lines to produce an F_1 generation of 120 animals. From this F_1 generation 7 males and 28 females were mated to produce in total 630 F_2 hens. The F_2 population was described in detail by Rodenburg *et al.*, (2003) and Buitenhuis *et al.*, (2003).

Housing Conditions

The F_2 hens arrived at the experimental farm as day-old-chicks in 5 batches at 2 weeks intervals. The birds were not beak-trimmed and each individual bird was marked with a wing-band. Each batch was divided over 2 pens, giving a total of 10 groups (batch x pen) with an average of 63 birds per group. The floor area of the pen was 4.75 x 2 m and covered with wood-shavings. Each pen had 2 light tubes (2 x 40W) and during wk 0 to 4 a heating lamp. From wk 0 to 4 there was continuous light from the heating lamp, while from week 5 to 6 the scheme was changed to 8-h light per day from 0800 to 1600 h.. From 16 weeks of age onwards the light scheme was extended with 1 h per week, until the animals had 16-h light a day from 0300 to 1900 h at 24 weeks of age. Feed and water were provided ad libitum (Rodenburg *et al.*, 2003; Buitenhuis *et al.*, 2003).

Phenotypic Traits

All F_2 birds were tested individually at an age of 5 (n=625) and 29 (n=550) weeks in the open-field test for 10 minutes. The open-field was an observation pen (1.25 x 1.25 m), divided in 25 squares of 0.25 x 0.25 m each. Each bird was picked randomly from its pen and placed in the middle of the open-field. The behavior was recorded using a camera. The observer recorded the behavior in an adjacent room from a video-screen. The behavior was recorded according to an ethogram described in detail by Rodenburg *et al.*, (2003). In the open-field test 9 traits were recorded using focal sampling: Duration of sitting (s), duration of standing (s), latency to stand up (s), latency to the first step (s), latency to the first call (s), frequency of steps, frequency of calls, frequency of flying and frequency of defecation. There

is a difference in vocalization in young birds and adult birds; at 5 weeks of age distress calls [Peep] and on 29 weeks of age alarm calls [Kot kot kot kodeeek] and alarm call notes [Kot] were measured. A detailed description concerning the distribution, averages and standard deviation of the traits were presented in an earlier paper (Rodenburg *et al.*, 2003). A factor analysis was performed on the 9 traits measured in the open-field using PROC FACTOR (SAS version 6.12; 1996), which resulted in only one factor with an Eigenvalue >1. Before performing the factor analysis, traits that were recorded as frequencies were transformed using a square-root transformation. Latencies were not transformed because the log-transformation had no effect on the distribution. All locomotion and vocalization traits loaded highly on one single factor. This factor was therefore called 'FEAR'. For each bird, a factor score was calculated and used in the QTL analysis.

Genotypic Data

Blood was taken from the wing veine from all grandparents (F_0), parents (F_1), and F_2 offspring at 5 week old birds. DNA was extracted according the Capture Plate^{TM} Kit protocol (Gentra Systems). All birds, from the F_0, F_1, and F_2-generation were genotyped with in total 180 micro-satellite markers evenly distributed in the genome as described by Buitenhuis *et al.* (2003). The amplification reactions were performed as described by Crooijmans et al. (1997). The PCR program used was: 5 min denaturation at 95 °C, 36 cycles of 30 s at 95 °C, 30 s at annealing temperature, and 30 s at 72 °C followed by a final elongation step of 4 min at 72 °C. The markers were run on an ABI373 sequencer (Applied Biosystems) and fragment sizes were calculated relative to the GENESCAN-350 TAMRA (Applied Biosystems) marker with GENESCAN 2.1 (Applied Biosystems) fragment analysis software and allele identification was performed using GENOTYPER 2.0 software (Applied Biosystems). The markers were covering 24 chromosomes and three additional linkage groups (26 autosomes and one sex chromosome (Z)) covering approximately 80% of the genome (Groenen *et al.*, 2000). The mapping distances reported are according the consensus linkage map, because the distances in this cross did not differ much from the consensus linkage map (Buitenhuis *et al.*, 2003).

Total Genome Scan

Prior to the QTL analysis, fixed effects were tested for significance. Only PEN (group in which the bird was housed) and TIME (time of testing) were significant for both ages. Therefore, data on the observations at 5 weeks of age were adjusted for PEN (j = 1,...,10) and

TIME (j = 1,…, 4) and at 29 weeks of age data on the observations were adjusted for PEN (j = 1,...,10) and TIME (j = 1,…, 4) using the PROC GLM procedure (SAS version 6.12; 1996).

For the QTL analysis a regression interval method was applied using two different genetic models: The paternal half-sib model (Knott *et al.*, 1996; De Koning et al., 1999) and the line-cross model (Haley et al., 1994; De Koning et al., 2000). In the half-sib analysis no assumptions were made concerning the allele frequencies and number of QTL alleles in the founder lines. The F_2 animals were treated as 7 unrelated half-sib families using the model:

$$Y_{ij} = m_i + b_i p_{ij} + e_{ij}$$

where Y_{ij} = trait measured on animal j, from rooster i; m_i = average of half-sib family i; b_i = substitution effect for a putative QTL; p_{ij} = the conditional probability for animal j inheriting the first paternal haplotype; e_{ij} = residual effect. In this analysis the contrast between the two haplotypes of every F_1 rooster was made. Analyses within families were performed and the test statistics was calculated as an F ratio for every centi Morgan (cM) on the chromosome (De Koning et al., 1999).

In the line-cross model the alternative alleles at the QTL were traced back to the original HFP and LFP lines. In general, it is assumed that the alleles in the founder lines are fixed but this is not an absolute condition. However, the power to detect QTL using the line-cross analysis drops rapidly when the alleles in the founder lines are not fixed (De Koning *et al.*, 2002). At every cM across the genome the extended model of De Koning *et al.*, (2000) was fitted:

$$Y_j = m + a_{pat} p_{patj} + a_{mat} p_{matj} + d p_{dj} + e_j$$

where Y_j = trait measured on animal j; m = population mean; a = additive effect; d = dominance effect; p_{pat} = conditional probability that an animal inherited a HFP allele from the sire ($[p_{11} + p_{12}] - [p_{22} + p_{21}]$); p_{mat} = conditional probability that an animal inherited a HFP allele from the dam ($[p_{11} + p_{21}] - [p_{22} + p_{12}]$); e_j = residual error. The model of De Koning *et al.*, (2000) is the same as the model of Knott *et al.*, (1998) in terms of total variance explained by the model. The test statistics was calculated as an F ratio for every cM along the chromosome. QTL with possible parent-of-origin effects were subjected to an additional test.

In this test it was tested if the imprinted model explained the observations better than a mendelian model (Knott et al., 1998).

The suggestive significance level and the genome-wide significance level were used according the guidelines of Lander and Kruglyak (1995). To determine the significance threshold for each trait individually, 10,000 permutations were performed on the data using a permutation test (Churchill and Doerge, 1994).

The ethical committee on animal welfare of Wageningen University, The Netherlands approved this study. The committee has judged the welfare of the animals within the scope of the experiment according to the Dutch law on the use of laboratory animals.

RESULTS

In Table I the QTL detected using the half-sib analysis and the line-cross analysis are presented for the traits measured at 5 weeks of age ordered by chromosome. In the half-sib analysis, a genome wide significant QTL was detected on GGA2 between marker brackets MCW0087 and ROS0023 for frequency of stepping at 5 wk of age (Table I). The within family analysis showed that one family (Family 1) out of the 7 families, contributed evidence for the presence of a QTL. The allele substitution effect in this family was 85.57 (number of steps) and the maximum F ratio was 16.07. For frequency of defecation, a suggestive QTL was detected on GGA11 between marker brackets LEI0143 and ADL0123. Here three families out of seven families (family 2, 6, and 7) contributed evidence for the presence of a QTL. Family 2 had an allele substitution effect of –0.52 (number of defecations) and an F ratio of 4.35 and family 6, which had an allele substitution effect of -0.46 and an F ratio of 4.02. Family 7 contributed most to the QTL with an allele substitution effect of 3.76 and an F ratio of 8.37. Using the individual factor scores (factor FEAR) in the QTL analysis, no QTL was detected using the half-sib analysis. The behavioral traits measured in the open-field at 29 weeks did not reveal a QTL using the half-sib analysis.

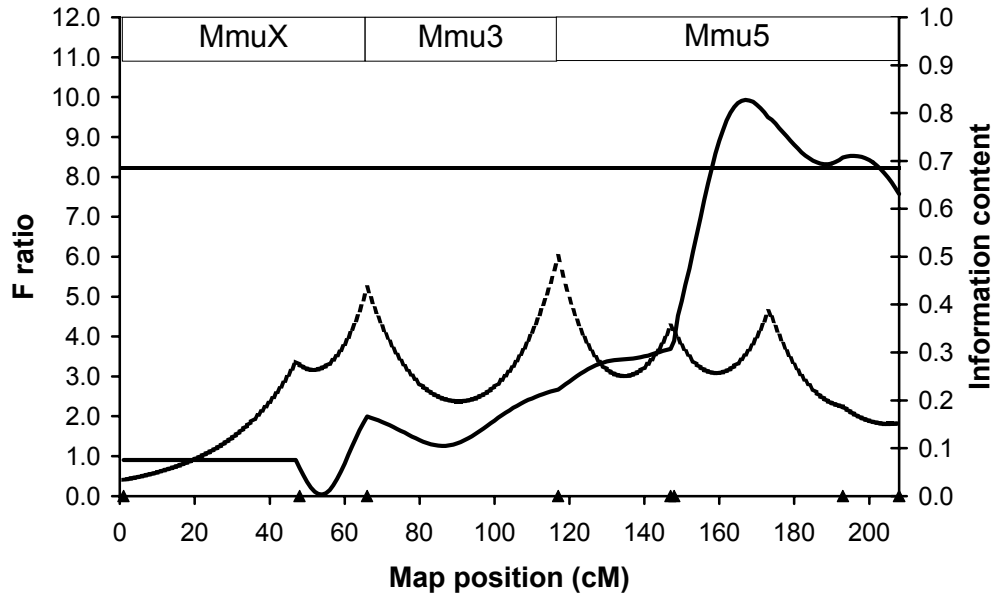
TABLE I. Quantitative trait loci affecting open-field behavior at 5 weeks of age using the line cross analysis.

Chr.	Trait	Pos. (cM)	Marker Bracket	a (SE)	d (SE)	F	
GGA1	latency time to calling	212	MCW0112-LEI0071	18.0 (18.7)	166.5 (43.6)	7.74	*
GGA2	*frequency of stepping*[1]	269	MCW0087-ROS0023	-	-	6.03	**
GGA2	latency time to walking	17	MCW0205-MCW0341	21.5 (12.3)	-114.9 (27.2)	10.45	**
GGA2	latency time to standing	210	GCT0020-MCW0042	-44.9 (20.6)	201.1 (58.0)	8.08	*
GGA2	factor FEAR	213	GCT0020-MCW0042	0.2 (0.1)	-0.9 (0.3)	8.32	*
GGA4	latency time to calling	193	MCW0180-MCW0174	82.3 (22.8)	45.9 (60.1)	7.32	*
GGA4	latency time to standing	168	LEI0081-MCW0180	46.0 (15.6)	138.1 (40.6)	11.19	**
GGA4	latency time to walking	167	LEI0081-MCW0180	22.2 (12.4)	116.4 (32.8)	8.61	**
GGA4	frequency of stepping	173	LEI0081-MCW0180	-8.6 (5.9)	-56.7(13.5)	10.86	**
GGA4	factor FEAR	167	LEI0081-MCW0180	-0.2 (0.1)	-0.7 (0.2)	9.92	**
GGA9	frequency of call	3	ROS0078-GTC0016	-146.2 (40.9)	-948.2 (237.5)	10.92	**
GGA11	*frequency of defecation*[1]	1	LEI0143-ADL0123	-	-	3.25	*

[1]QTL detected using the half-sib analysis. For each trait measured in the open-field test the chromosome (Chr), the estimated position (Pos) on the chromosome, the marker bracket, the additive and dominance gene effects (standard error) and the F-statistics (F) are given. * denote suggestive linkage, ** denote genome wide significant linkage at the 5% level.

Analysis of the behavioral data using the line-cross analysis revealed several QTL for both open-field behavior at 5 weeks of age (Table I) as well as for open-field behavior at 29 weeks of age (Table II). QTL detected for the same traits at different ages do not show any overlap. At 5 weeks of age, a genome wide significant QTL on GGA4 between marker brackets LEI0081 and MCW0180 was detected for locomotion traits (latency time to standing, latency time to walking, frequency of stepping). Using the factor FEAR from the factor analysis, this QTL on GGA4 was also detected. In Figure 1, a graph is shown of the test-statistics for the genome-wide significant QTL on GGA4 for factor FEAR at 5 weeks of age. The information content of the first marker used on GGA4 (ADL0203) is very low. The test-statistics could not be calculated properly and resulted in a flat line. On GGA2 a genome wide significant QTL between marker brackets MCW0205 and MCW0341 was detected for latency time to walking, but was not detected when using the

FIGURE 1. QTL for factor FEAR at 5 wk of age on GGA4. The F ratio profile is denoted as a solid line and the information content is denoted as a dashed line. The horizontal line denote the 5% genome wide significance threshold. The chicken-mouse comparative map is given in the upper bar.



factor analysis. For frequency of calling, a genome wide significant QTL was detected on GGA9 between marker brackets ROS0078 and GCT0016. When using the individual factor FEAR in the QTL analysis, there was no statistical evidence for a QTL on GGA9 for frequency of calling.

At 29 weeks of age, a genome wide significant QTL was detected between marker brackets ADL0106 and LEI0103 for the trait latency time to calling (Table II). For the trait latency time to walking genome wide significant QTL were detected on GGA1 between marker brackets MCW0106 and ADL0124 and on GGA4 between marker brackets MCW0114 and LEI0094. These QTL were also detected using the factor FEAR from the factor analysis. In addition, suggestive QTL were detected based on the separate traits. Figure 2 shows a graph of the test-statistics for the genome-wide significant QTL for factor FEAR at 29 weeks of age located on GGA4. On GGA1, three different QTL were detected for the traits latency time to calling, frequency of calling and latency time to standing, respectively (Table II). And one genome wide significant QTL was detected on GGA3 for latency time to standing. These QTL were not detected using the factor FEAR. In general, QTL at 29 weeks of age are smaller in magnitude compared to QTL at 5 weeks of age. The dominance component for the behavioral traits measured for both ages were very high, suggesting over-dominance.

FIGURE 2. QTL for factor FEAR at 29 wk of age on GGA4. The F ratio profile is denoted as a solid line and the information content is denoted as a dashed line. The horizontal line denote the 5% genome wide significance threshold. The chicken-mouse comparative map is given in the upper bar.

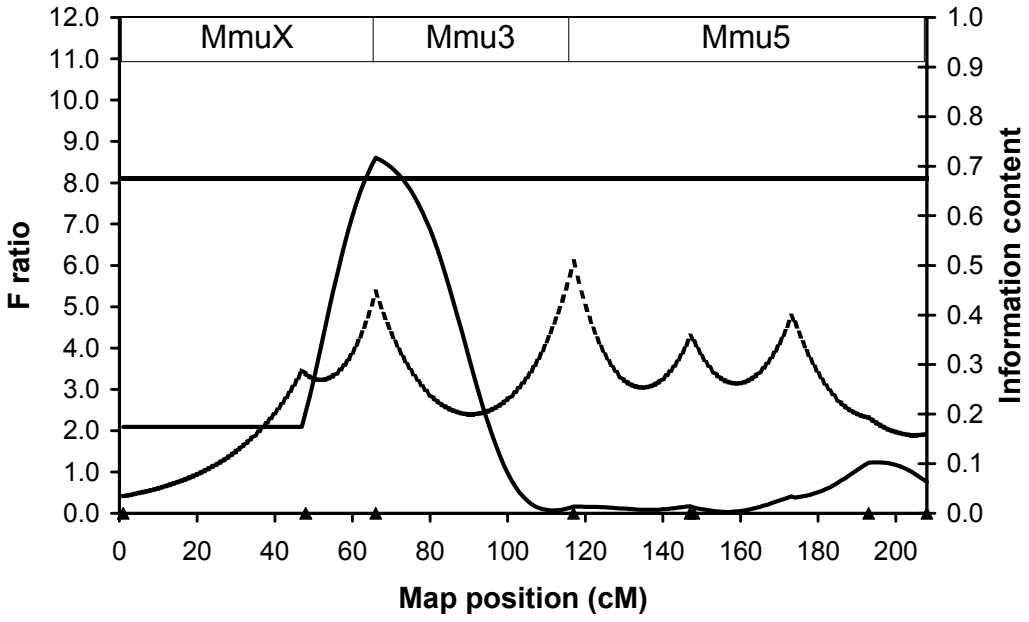


TABLE II. Quantitative trait loci affecting open-field behavior at 29 weeks of age using the line cross analysis.

Chr.	Trait	Pos. (cM)	Marker bracket	a (SE)	d (SE)	F	
GGA1	latency time to calling	536	LEI0134-MCW0107	-63.9(17.4)	-59.1(44.9)	7.28	*
GGA1	frequency of calling	319	MCW0109-MCW0046	-62.1(17.0)	-75.9(53.0)	6.91	*
GGA1	latency time to standing	40	MCW0346-LEI0194	57.0(29.2)	269.2(75.8)	6.91	*
GGA1	latency time to walking	111	MCW0106-ADL0124	-46.8(13.4)	-120.9(33.5)	7.93	*
GGA1	factor FEAR	106	MCW0106-ADL0124	0.3(0.1)	0.8(0.3)	7.54	*
GGA3	latency time to standing	170	MCW0004-MCW0059	-66.9(29.1)	-196.2(69.3)	12.40	**
GGA4	latency time to calling	67	MCW0114-LEI0094	47.8(13.8)	57.9(34.7)	7.76	*
GGA4	latency time to walking	66	MCW0114-LEI0094	36.2(8.9)	36.5(21.5)	10.02	**
GGA4	factor FEAR	66	MCW0114-LEI0094	-0.3(0.1)	-0.3(0.2)	8.61	**
GGA10	latency time to calling	101	ADL0106-LEI0103	-41.0(13.2)	77.6(27.0)	9.09	**
GGA10	factor FEAR	101	ADL0106-LEI0103	0.2(0.1)	-0.4(0.1)	7.39	*

For each trait measured in the open-field test the chromosome (Chr), the estimated position (Pos) on the chromosome, the marker bracket, the additive and dominance gene effects (standard error) and the F-statistics (F) are given. * denote suggestive linkage, ** denote genome wide significant linkage at the 5% level.

DISCUSSION

In the current study, we have mapped QTL for open-field behavior in young and adult chickens. Two commercial white leghorn lines both selected on egg production traits, but differing in behavioral traits (Jones *et al.*, 1995; Rodenburg and Koene, 2003) were used to generate a QTL mapping population.

Data on open-field behavior at 5 and 29 weeks of age were analyzed using a half-sib analysis (Knott *et al.*, 1996) and a line-cross analysis (Haley *et al*., 1994; De Koning *et al*., 2000). In general, analyzing the data with the line-cross model revealed more, and different, QTL than using the half-sib analysis. In the line-cross model the alleles are traced back to the founder lines. When the loci are fixed at alternative alleles in the founder lines, the power to detect a QTL using a line-cross analysis is considerably higher than when the alleles are segregating in the founder lines (De Koning *et al.* 2002). In case the alleles are segregating, the half-sib analysis might be more appropriate and powerful to analyze the data. The founder lines used to generate our F_2 population have been selected for egg production traits but were not selected directly for open-field behavior. However, when open-field related behavior traits are highly correlated with egg production traits, QTL alleles might be fixed or close to fixation in the founder lines. In the case of no, or a low, correlation between the production trait selected for and open-field behavior, the QTL alleles may still be segregating. The advantage of using two different methods to analyze the data was that both methods reveal different QTL. In our study, no statistical evidence for parent-of-origin effect was detected using the model proposed by De Koning *et al.*, (2000).

The traits scored in the open-field are highly correlated at a young age (Rodenburg *et al.* in prep.). To put the different behavioral observations in order, a factor analysis was performed. The factors obtained from the analysis are independent from each other. A QTL analysis on these separate factors should result in QTL underlying these factors analogous to Weller *et al.,* (1996). However, a QTL analysis on the separate traits, underlying one factor, revealed additional QTL, which were not found using the factor scores. This indicates that, ordering the behavioral phenotypes using factor scores can be very helpful to cluster correlated traits, but it may increase chances of missing additional QTL.

The QTL detected for open-field behavior at 5 weeks of age were not the same as the QTL detected at 29 weeks of age, which might indicate that different genes are involved in behavior at different ages. In the open-field test the birds were observed individually, i.e.

removed from their social group. Therefore, the underlying motivation of a specific behavior might change with age, e.g., an older animal may be able to know better how to deal with the situation, and the behavioral pattern in a certain situation, although similar between ages, may involve different genes. It is, for example, known that the social hierarchy of the group in which chickens are housed must be established within the first 5 weeks of age (Guhl, 1958). During the first three weeks, an ordered group structure is maintained for the top-ranking birds, but in the low-ranking birds the group structure is less stable (Rogers and Astiningsih, 1991). The difference in the social group structure between young and adult age may influence motivation to reinstate social contact and can, therefore, influence behavior in the open-field (Gallup and Suarez, 1980). The different QTL found between ages for open-field behavior clearly suggest that there were different genes involved in behavior at different ages. This was in agreement with the study of Rodenburg *et al.* (in prep.). Although there is a relation between young and adult hens in open-field behavior, e.g., inactive open-field behavior at young age is correlated with active open-field behavior in adult hens, there are a number of behaviors at young age which do not show a correlation between ages. Therefore, the study of Rodenburg *et al.* (in prep.) and this study support the idea that behavior measured on different ages should be considered as different traits that are regulated by different genes. These findings were also consistent with observations on feather pecking behavior (Buitenhuis *et al.* 2003) and receiving pecks (Buitenhuis *et al.* submitted) where QTL detected between ages do not coincide.

The QTL found for open-field behavior in mice (Flint *et al.*, 1995; Turri *et al.*, 2001 a,b) may be of value in helping to identify possible candidate genes involved in open-field behavior in chickens. Candland and Nagy (1969) showed that when a chicken is placed in the open-field, its behavior is characterized by freezing and reduced vocalization. When coping styles, as described by Koolhaas *et al.* (1999) are used to compare chickens, rats or mice, the initial behavioral response of a chicken in an open-field can be compared behaviorally with a "reactive" rat or mouse. In the lineage leading to mammals and birds, the chicken diverged from the common ancestor 300 Myr ago (Burt *et al.*, 1999). Due to this time-span, the genetics involved in the regulation of behavior may, or may not, have evolved differently between mice and chickens.

From the current study, it seems likely that the genes involved in open-field behavior in the chicken are different from the ones involved in open-field behavior in mouse. Based on a comparative map between them (Burt, 2002), it is clear that, in general, the QTL regions for

open-field behavior in chicken do not match the mouse chromosomes where QTL have been identified for open-field behavior (Flint *et al.*, 1995; Turri *et al.*, 2001a,b). An exception, however, may be GGA4, because two QTL were detected on this chromosome, one QTL at 5 and one QTL at 29 weeks of age. Because the estimated QTL positions were more than 100 cM apart it was not likely that one gene affecting both ages is involved.

Fine-mapping of the QTL for open-field traits in subsequent generations, should result in narrowing down the QTL regions involved. The QTL region for open-field behavior at 5 weeks on GGA4 shows homology to Mmu5 (Schmid *et al.*, 2000). The QTL region for open-field behavior at 29 weeks, has a region which is homologous with MmuX (Schmid *et al.*, 2000; Burt, 2002). In mice, open-field activity on the first day of testing, behavior on the elevated plus maze test and in the mirror chamber were strongly correlated with MmuX at 50 cM (Turri *et al.,* 2001a). MmuX was also associated with open arm entries (16 cM) and time spent in the open arms of the elevated plus maze (0 cM) (Turri *et al.,* 2001b). In chickens, 6 genes were mapped which show homology to MmuX (17 cM to 51 cM) (Schmid *et al*., 2000). In Figure 1 the chicken – mouse comparative map is given between GGA4 and MmuX, Mmu3, and Mmu5. In the next phase of analysis more informative markers are needed to deal with the low information content on the beginning of GGA4. This will likely result in a better estimate of the QTL position. To date, a dense comparative map of GGA4 with humans and mice is under development using the Wageningen chicken BAC library and the chicken radiation hybrid panel (Rabie *et al.,* in preparation). The ensuring information information on the order of genes may provide insight as to where the actual breakpoint between MmuX and Mmu3 is situated, which will be essential to decide whether the same genes are influencing open-field behavior in chickens and mice.

In the present study, we are the first to report the identification of QTL regions for open-field behavior at two different ages in chickens. The QTL regions do not coincide between ages, indicating that behavior at different ages is regulated by different genes.

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CHAPTER 7

GENERAL DISCUSSION

The objective of the study described in this thesis is to explore the genetic basis of feather pecking in chickens. In a later stage of this research the exploration of the behavioral repertoire was not restricted to feather pecking but extended to open-field behavior. Different (genetic) approaches were used: heritabilities of behavioral traits (Chapter 2) were estimated, and QTL involved in (feather) pecking and open-field behavior (Chapter 3, 5, 6) were mapped. Furthermore, a comparative chicken-human gene map was made between GGA13 and HSA5 (Chapter 4), because candidate genes for behavioral traits are located on HSA5. Comparative mapping will give more precise insight in the location of candidate genes on the chicken genome.

The general discussion consists of five parts. The first part deals with the definition of the phenotype. In the second part the results of the QTL analysis in relation to the hypothesis of Korte et al. (1997) is discussed (Chapter 3 and 4). The third part deals with the candidate gene approach. The fourth part deals with the possibilities to go from QTL to gene. In the fifth part the possibilities to reduce the feather pecking (FP) problem in practice are discussed from a genetic point of view.

Definition of the phenotype

The observed phenotype is dependent on the genetic background of an animal and the environment in which it is situated. The performance of an animal may be improved when the environment is improved, e.g., when higher quality feed is provided. By comparing animals under different environmental circumstances, one may observe that genotype A is superior to genotype B in environment X, but inferior in environment Y (Falconer and Mackay, 1996). This is referred to as genotype by environment interaction (GxE). A nice example of GxE for behavioral traits is shown in the study Crabbe et al. (1999). In this study eight different inbred mouse strains were characterized for eight different behavioral tests in three different laboratories. Despite the extensive fine tuning of the testing protocols used at the different locations, large effects of the environment were detected. However, for some behaviors like ethanol drinking, laboratory environment was not critical.

With regard to FP behavior, it is shown that environmental influences can play an important role in the initiation of pecking behavior (Chapter 1). It was also shown that when there was full light and no wood shavings available on a slatted floor, there was no difference in FP behavior, i.e., there was no visible difference between the plumage condition of the LFP or HFP line (Rodenburg et al., 2002). Adjusting the environmental conditions by providing

wood shavings and dimming the light, the differences between the LFP and HFP line became discernable again.

The example above indicates that chickens with different genetic backgrounds perform differently in the same environment, however, modifying the environment can diminish the difference. Therefore, a reliable FP test is to be preferred, i.e., when a bird is characterized as non-pecker, it should show this behavior under a wide spectrum of environmental circumstances. Different tests have been used to estimate genetic parameters (Chapter 1). Rodenburg and Koene (2003) have evaluated different tests and concluded that the social FP test was the test which represented FP behavior in the group best. Because pecking behavior is performed in a social context, measuring this trait in a group situation would be preferred (Rodenburg and Koene, 2003). The social FP test was already used in the study of Kjaer and Sørensen, (1997) and Kjaer et al., (2001). These studies showed that the social FP test was useful for estimating genetic parameters (Kjaer and Sørensen, 1997) as well as for selection for FP behavior (Kjaer et al., 2001). Using the social FP test for characterization of the F_2 population revealed heritabilities in the same range as found by Kjaer and Sørensen, (1997). In addition, it was possible to identify QTL for different forms of pecking as well as for being pecked (Chapter 3 and 5). These results create the prospect the FP problem might be reduced using conventional methods.

With regard to the example of GxE interaction for behavior. It would be of interest to study whether GxE interaction does actually exist for FP behavior. Thus it would be worth knowing whether the social FP test would be useful when applied under more harsh environmental circumstances. The availability of a reliable test for FP behavior is not only important to validate QTL in subsequent generations in an experimental set up as a follow up of this study, but also for the use in practice under commercial conditions.

Corticosterone in relation to FP behavior

Corticosterone is a glucocorticoid hormone playing a role in the stress response. Therefore, the concentration of corticosterone in the blood is often used as a measure of stress. In human under normal circumstances, corticosterone levels show a clear pattern of circadian rhythmicity, i.e., high early in the morning with a decline towards the evening (Horrocks et al., 1990). When considering stress response as a measure of stress one can distinguish an acute stress response and a chronic stress response. During an acute stress response the plasma corticosterone level in the blood increases and after a while the

concentration decreases. In case of a chronic stress response plasma corticosterone levels are maintained on a high level for a prolonged time period, e.g., when an animal is kept in a low stimulus environment.

In rodents one can distinguish different behavioral reactions to a stressful environment which coincides with a difference in corticosterone response. Reactions are either active or passive. Active animals show a low corticosterone level and passive animals show a high corticosterone level (Koolhaas et al., 1999; Chapter 1 of this thesis). Korte et al. (1997) showed that the HFP line had a consistent lower corticosterone response to a manual restraint test than the LFP line, which indicates that the chickens of the HFP line and LFP line fit in the coping style theory according to Koolhaas et al. (1999). The observation of Korte et al. (1997) was confirmed by the study of Van Hierden et al. (2002), indicating that there might be a relation between a low corticosterone response and high FP. The best study to find out the relation between corticosterone and FP behavior is to estimate the genetic and phenotypic correlations between these traits. However, the sample size in the studies on the founder lines are too small to make a good estimation of genetic parameters (Korte et al., 1997; van Hierden et al., 2002). A correlation study between corticosterone and FP behavior in the F_2 population is currently underway and, therefore, the results could not be discussed here. When a low corticosterone response is accompanied by high FP, this might be explained at the gene level by close linkage or pleiotrophy, i.e., the same gene influence two different traits. A QTL study will not reveal an answer on whether there is close linkage or pleiotrophy, but may indicate that such phenomena play a role.

The data on the manual restraint test were used in a QTL study to explore the genetic basis of the acute stress response in chickens (Chapter 3). The QTL analysis revealed two QTL: one chromosome wide QTL on GGA15 and one suggestive QTL on GGA18 (Chapter 3). The QTL identified for stress response do not coincide with the QTL detected for gentle FP (GGA2) and severe FP (GGA2) behavior at 30 wk of age (Chapter 3). This shows that there is no indication of close linkage or pleiotrophic effects playing a role between corticosterone and FP behavior. This finding is supported by experimental research in rats where lack of association was found between physiological parameters and behavior: Hendley et al. (1986) showed that hypertension and hyper activity were no longer correlated between Spontaneously Hypertensive Rats and Wistar Kyoto rats. In the same manner neuroendocrine measures, general activity, and anxiety related behaviors were associated at the strain level in

Wistar Kyoto rats and Wistar Kyoto Hyperactive rats but segregated independently in an F_2 population (Courvoisier et al., 1996).

From the literature the relation of corticosterone (stress) with FP behavior is not directly clear. In a study of El-Lethey et al. (2000), different indicators for stress were studied in relation to FP. It was concluded that FP was associated with (chronic) stress - the study was conducted over 12 weeks - because foraging material and food form had an influence on both FP behavior as well as on the stress indicators (heterophil/lymphocyte ratio, i.e., a measure for corticosterone level, tonic immobility test, and immune response to sheep red blood cells, tetanus toxoid and human serum albumin). In another study by El-Lethey et al. (2001), supplementation of corticosterone to chickens in the feed suggested that corticosterone can trigger FP. However, hens kept on slats and not fed with corticosterone showed similar frequencies of FP. This indicates that corticosterone can play a role in FP behavior, but is not a causal factor.

Based on the literature and the observations presented in this thesis it is remains probable that corticosterone plays a role in FP behavior. However, it can be concluded that the use of corticosterone response to manual restraint, which is an acute stress response, is not a useful predictor for feather pecking behavior.

Candidate genes

Understanding the biology of complex traits in model species offers an alternative to QTL analysis to identify genes responsible for traits of interest in farm animals. A genetic analysis of a trait based on candidate genes requires information on the biological pathways involved in the trait. Based on the available literature one can make the best possible guess which gene(s) are likely to be involved in the trait of interest. Association between a candidate gene and trait could assign biochemical pathways to a certain trait, which would be helpful to unravel the molecular principle underlying the trait.

To find a-priori candidate genes a literature survey was conducted and five candidate genes which influence behavior in mice (Serotonin receptors (HTR1A; HTR4), Dopamine receptors (DRD1; DRD3) and the Glucocorticoid receptor (NR3C1) and Mineralocorticoid receptor (NR3C2)) were identified. These genes are playing a role in signal transductions in neurons or regulate the stress response, therefore these genes could also play a role in FP behavior. At the beginning of this project (March 1999), the chicken consensus linkage map and gene maps were still under construction. It was almost a year later when the “First report

on Chicken Genes and Chromosomes 2000" was reported (Schmid et al., 2000), including a rough chicken-human comparative gene map.

Because the above mentioned candidate genes were not identified in chicken, the chicken orthologous genes had to be identified. Based on the comparison of the exon-intron structure of the candidate genes between different species, primers were designed in the most conserved regions, because it is likely that this would be conserved in chicken too. However, this approach did not result in clear bands after PCR amplification in chicken, indicating that a-specific amplification was troubling the identification of the genes. This is also observed when using comparative anchor tag sequences (CATS)-primers, where between mammalian species genes can be amplified using the same primers, however, these primers do not work or could not be optimized in chicken (Lyons et al., 1997). In addition, hybridization screening of a chicken embryo and brain EST library with human cDNA clones resulted in a-specific binding of the human probe and chicken cDNA clone. Therefore, a different approach was used by making a comparative chicken-human map. Three candidate genes were located on human chromosome 5 (HSA5) (HTR1A: HSA5q11.2-q13; HTR4: HSA5q31-q33; and NR3C1: HSA5q31). Based on the comparative map (Schmid et al., 2000) GGA13 was homologous to HSA5 in human, therefore it was decided to make a detailed comparative map between HSA5q31 and GGA13 as described in Chapter 3. The sequencing of chicken Expressed Sequence Tags (EST) e.g., http://www.chick.umist.ac.uk/ or the DT40-cell line (Abdrakmanov et al., 2000) reveal a lot of new information on genes in the chicken. By now the chicken orthologs have been identified for a D1-like receptor (L36877; L36878; L36879), the glucocorticoid receptor: NR3C1 (AY029202; AF525751), and the mineralocorticoid receptor: NR3C2 (AF468211). The serotonin receptors, however, have not been identified yet.

GGA13 did not show up in the QTL analysis for both corticosterone response as well as for the FP behavior, which makes NR3C1 a less likely candidate gene for either traits. Although the candidate-gene approach method could be promising, this approach has been subject to two important criticisms. First, the significant findings of association in many candidate-gene studies have not been replicated when followed up in subsequent association studies. E.g., in pigs it was reported that the MC4R gene has an effect on fatness, growth and feed intake traits (Kim et al., 2000), however, the effect of the MC4R gene on fatness traits could not be validated in a different mapping population (Park et al., 2002). Second, because candidate-gene studies are based on the ability to predict functional genes and variants, some

critics argue that current knowledge is insufficient to make these predictions (Tabor et al., 2002).

Nevertheless, the development of a comparative mapping with the well-developed human and mouse gene maps could be an important tool in the process of identification of corresponding genomic regions or major genes controlling traits of interest. In addition, comparative mapping between chicken and human of GGA13 and other chromosomes (GGA10: Crooijmans et al. (2001); GGA15: Jennen et al. (2002); GGA24: Jennen et al. (2003); and GGA28, GGA11: Smith et al., 2002) show that there are multiple intra- and extra-chromosomal rearrangements between chicken – human and mouse. The structure of the re-arrangements between these species in the QTL region is of importance for the identification of candidate genes based on the literature available on behavior in relation to genes.

A QTL and then?

The area of QTL mapping of behavioral traits in farm animals is relatively new. To date (May 2003) there are only a few studies mentioned for pigs (Désautés et al., 2002), cows (Schmutz et al., 2002), honey bees (Hunt et al., 1998; Lapidge et al., 2002; Arechavaleta-Valasco et al., 2003), and chicken (Schütz et al., 2002; Buitenhuis et al., 2003a,b,c) dealing with behavior. In the search for the genes influencing the trait of interest, a QTL study is only a first step.

Founder lines

A QTL study starts with making a choice of the founder lines. QTL studies in chicken make use of different founder lines for the QTL crosses, e.g., layer-layer (Tuiskula-Haavisto et al., 2002; the study reported in this thesis), broiler-broiler (Van Kaam et al., 1998), and broiler-layer (Sewalem et al., 2002) crosses. In addition, crosses of unrelated lines (Schütz et al., 2002), specific selection lines (Siwek et al., 2003b), and inbred lines (Zhou et al., 2001) are reported. With the same number of animals, the advantage of using inbred lines in dissecting the genetic basis of complex traits is that the power to detect QTL affecting the trait of interest is higher compared to a study using a cross of non-inbred lines. The same principle holds for the use of selection lines. The lines used in the study described in this thesis, are commercial White Leghorn lines which were selected for egg production traits and not selected for behavioral traits. Nevertheless, these lines do differ consistently in their propensity to FP (Rodenburg and Koene, 2003). This difference can be due to random drift or

due to a correlated response. The relation between FP behavior and production traits is not clear and would deserve more attention. Nevertheless, QTL mapping experiments on traits not directly selected for can be very useful as shown in the study of Siwek et al. (2003a), in which a QTL was confirmed influencing the primary immune response to keyhole lympet hemocyanin in two unrelated populations.

Mapping strategy

In choosing the appropriate strategy for a QTL-mapping experiment several criteria are of importance, e.g., the power of the alternative designs, the dominance state of the genetic effects, and resources available, e.g., laboratory equipment and animals (Darvasi, 1998). For an initial genome scan the two experimental designs mostly used are the backcross (BC) and the intercross (F_2). In a BC the F_1 is backcrossed to one of the founder lines. In an F_2 design the F_1 is intercrossed to generate the F_2 animals. For studying the general picture, i.e., number of QTL segregating and estimates of the additive and dominance effects, an F_2 design is preferred because if there is complete dominance for an allele coming from founder line (A), a BC of the F_1 animal with founder line A will result in a power of zero at any sample size (e.g., Belknap and Atkins, 2001). In the study reported in this thesis there was no indication on possible dominance or known gene effects concerning feather pecking behavior. It was well documented that the lines consistently differed for the traits of interest (Blokhuis and Beutler, 1992; Jones et al., 1995; Korte et al., 1997; Van Hierden et al., 2002; Rodenburg and Koene, 2003) . In such a situation an F_2 design is the most appropriate design for an initial QTL study because this will give an indication whether there is a QTL segregating and one can estimate the additive and dominance effects (Darvasi, 1998).

Fine mapping

When the chromosomal fragments have been identified as being correlated with the phenotypic variability of the trait the major goal will shift to identification of (major) gene(s) involved in the variation of the trait. An initial QTL study typically provides an estimate of the QTL position with a confidence interval (CI) of approximately 50cM. In genetic terms this region is far too big to identify candidate genes. In order to refine the QTL region, fine-mapping of the QTL region is necessary. Fine-mapping of the QTL region consists of two steps. The first step is to type additional marker in the QTL region of interest. Because in the F_2 generation the number of informative meiosis are limited, the linkage disequilibrium

regions will not become smaller. The necessary second step is to reduce the CI due to crossing-over by the generation of subsequent generations. This can be achieved by generating an advanced intercross line (Davarsi and Soller, 1995). After 9 to 10 generations there is no extra gain anymore to narrow down the CI. However, the resolution obtained after 9 to 10 generations is not small enough to identify a causal gene. An additional disadvantage of this approach might be that when a QTL consists of multiple genes these genes break apart and the QTL can not be detected in a subsequent generation because the effect of the separate 'new' QTL are too small to detect (Darvasi and Soller, 1995). The next step in trying to get a better resolution is the definition of linkage disequilibrium blocks. The completion of the human and mouse genome sequencing projects have revealed conserved regions in the genome where crossing-over is low (Dawson et al., 2002; Wiltshire et al., 2003). For fine-mapping those linkage disequilibrium blocks are the highest resolution one can get, assuming there is no limit in the number of genetic markers available. In theory this fine-mapping can be achieved by generating single nucleotide polymorphism (SNP) markers. This type of markers is more abundant throughout the genome than the microsatelitte markers and is very useful for the detection of linkage disequilibrium patterns (Shifman et al., 2003). In chicken a frequency as high as one SNP per 100bp has been observed (Vignal et al., 2000). Most SNPs were identified by a direct sequencing approach of PCR-amplified DNA. Smith et al. (2001) used this approach to identify 65 SNPs in 17,500bp derived from 37 EST. In a similar approach 139 SNPs in 31,000bp were derived from 33 different genes and cosmids (Schmid et al., 2000). However, the exact number of SNPs to use and the number of chromosomes to type for the identification of haplotype blocks is still not clear (Cardon and Abecasis, 2003).

The F_2 mapping population from the HFP and LFP line is very valuable to study the genetic background of FP and open-field behavior. The F_2 animals have been extensively studied and heritablities, genetic and phenotypic correlations between behavioral traits were estimated (Chapter 2; Rodenburg et al., in prep.), and QTL were mapped for the different behavioral traits measured (Chapter 3, 5, and 6). Fine-mapping of the QTL in the F_2 generation still need some major improvement. A considerable effort should be made in the development of new genetic markers to design a saturated marker map. Typing additional markers on the F_2 population, will generate a better estimate of the QTL position. This may imply that the QTL peak may shift when additional markers are added (Siwek et al., 2003b). An additional advantage is the presence of an F_4 generation, which offers the possibility for extensive fine-mapping of the QTL found in subsequent generations and will facilitate the

identification of linkage disequilibrium blocks using SNP markers. Nevertheless, for the identification of genes there are some considerations and restrictions.

Considerations and restrictions

In mice identification of QTL for behavioral traits is rather successful, however, no genes representing the QTL have been identified so far. Most QTL identified do not have very large effects indicating that it is hard to find a major QTL (gene). Taking into account the genes underlying a QTL one can distinguish two types of mutations: functional mutations, i.e., the mutation causes a change in the protein, and mutations in regulatory elements, i.e., change the gene expression. Because the QTL for behavioral traits detected in mice are not very large, Flint (2003) suggested that these QTL represent only subtle changes in gene function or gene expression. These changes could well lie in the regulatory sequences, which may be at some distance away from the gene they influence (Flint, 2003).

New developments in technology like, gene expression profiling using DNA micro-arrays enables studying the activity of genes in different tissues and locations. Gene expression profiling may provide knowledge about the genes which are expressed at a certain time in a certain tissue. With regard to (FP) behavior, expression profiling of animals may present some difficulties but is challenging. There are a few considerations to make:

1. Selection of animals: The animals can be divided in peckers vs non-peckers coming from both the HFP and the LFP line.
2. The time point of measuring the gene expression: Once having a bird classified as a pecker or a non-pecker, the time point for measurement of gene expression is not obvious. There are a few possibilities: first the gene expression can be measured when birds in both groups do not show FP behavior. Second gene expression can be measured when birds in both groups do show FP behavior. The difficulty here is that one does not know what the optimal time is to measure relevant differences in gene expression.
3. Choice of tissues in which gene expression is measured. At this moment one does not know which tissue is crucial in the initiation of FP behavior. The brain is the prime candidate tissue to identify genes influencing FP behavior, however, other tissues may play a role as well.

Despite the difficulties raised above in the search for genes involved in FP behavior using expression profiling would be interesting. First of all, because it may validate the influence of a candidate gene in the trait of interest. Secondly, it may be more relevant for the

identification of new candidate genes. The same was successfully applied in a study for the expression of genes involved in breast cancer (Van 't Veer et al., 2002). However, whether gene expression profiling studies are helpful in the identification of genes underlying a QTL remains to be seen because the action of genes underlying a QTL can be more complicated than a difference in expression only. The complicated action of genes underlying a QTL involved in high-temperature growth was shown for yeast (Steinmetz et al. 2002). Yeast is an organism that is fully sequenced. Its genome is (relatively) easy to manipulate. Genome-wide expression profiling in combination with a QTL study shows that: "Functional linkage in both *in cis* and *in trans* were found between three tightly linked quantitative trait genes that are neither necessary nor sufficient in isolation". When the same applies to QTL in chickens (and mammals), it may turn out to be difficult, but challenging, to work out the actual influence of tightly linked genes is on the phenotype of study.

Animal model

To study the complexity of the gene action of the QTL one needs to have a good model. As a first step towards the study of gene function in mammalian genetics, a mutagenesis program in mice was developed to screen the whole genome for functional mutations (Nolan et al., 2000; Hrabé de Angelis et al., 2000). To produce new mutant lines male mice were injected with ethyl-nitrosourea and then used to produce F_1 animals. F_1 animals were analyzed for novel phenotypes of dominant or semi-dominant traits and bred further to screen for recessive phenotypes. This method has already revealed mutants influencing behavior, indicating that mutagenesis could be a valuable tool to study genes involved in behavioral traits (Nolan et al., 2000). For chicken such a method, however, is not feasible due to the long generation interval. It will take too much time to efficiently screen mutant chicken. However, to identify candidate genes in a particular chromosomal region it is mandatory to develop a strategy to manipulate a candidate gene in order to study its effect on the trait of interest. An alternative method (which has to be developed in chicken) could be gene silencing using interference RNA (iRNA), i.e., complementary RNA of the candidate gene which anneals to the mRNA, in a way that the mRNA cannot be translated. The amount of protein is reduced or absent and this may influence the trait of interest. iRNA has been proven to be a powerful tool in the elucidation of gene function in organisms ranging from worms, to plants and fruit flies (http://www.bio.com/newsfeatures/). However, due to the antiviral response of mammalian cells to dsRNA, the 'interferon response', the use of iRNA

in mammals has been complicated. Recently, the problem of the 'interferon response' has been overcome by Shinagawa and Ishii (2003). They have developed a new method to generate tissue specific knockdown mice by making a new vector to express long dsRNA from an RNA polymerase II promotor.

With regard to chickens, genome sequencing has started and would be a major help for the identification of candidate genes. The chicken is an important experimental system for developmental biology, immunology and microbiology (Brown et al., 2003) and several QTL studies have been performed for economical important traits. Utilization of the new technologies mentioned above would be a major improvement in studying the genetics of quantitative traits.

Possibilities to reduce the FP from a genetic point of view

Selection for reduced FP behavior has become of interest in Europe because in 2012 cage-systems will be prohibited as housing systems. Keeping chickens in alternative housing systems have their drawbacks such as higher disease incidence and mortality due to FP and cannibalism. From a commercial point of view it would be of interest to have animals that are well adapted to the group situation, i.e., animals which maintain production with a low sensitivity to the higher disease pressure, and with a low incidence of FP behavior.

Studies on the genetics of FP behavior have shown that FP is heritable (Cuthbertson, 1980; Bessei, 1984; Kjaer and Sørensen, 1997; Bessei et al., 1999). The heritability estimates range from 0.05 to 0.5, depending on the way the trait was measured (see for the different tests Chapter 1). Heritability estimates based on the F_2 population for gentle FP was 0.12 (0.07) at 6 weeks of age and 0.15 (0.08) at 30 weeks of age (chapter 2). For severe FP, however, heritability estimates were low. The heritability estimates are close to the estimates of Kjaer and Sørensen (1997), 0.13 (0.07) at 6 weeks of age and 0.13 (0.07) at 38 weeks of age. Kjaer and Sørensen (1997) did not separate the trait in different forms of FP as proposed by Savory (1995), but combined all forms of pecks, i.e., gentle and severe, in its heritability estimate. In both studies the same type of test (the social FP test) was used, indicating that testing birds in groups is a good and apparently a consistent measure to get a grip on FP behavior.

Although the heritability for FP behavior is low, it offers the possibility to select for reduced FP behavior in practice as shown in the study of (Kjaer et al., 2001). As selection criterion the 'number of bouts' was used (bout = repeated pecking directed at the same spot).

The trait ‘number of bouts’ does not distinguish between gentle and severe pecking, i.e., it is a combined trait for both gentle and severe pecking. It was possible to have significant difference in FP behavior after 3 generations. Interestingly there was no change observed in the level of aggressive pecking.

Direct selection against cannibalistic pecking can be successful using group selection, based on half-sib groups (sire family based selection) (Craig and Muir, 1993). They selected for hen-days without beak-inflicted injuries for 168 days in the age of 16 to 40 weeks based on sire family averages. Already after two generations the lines differed significantly. The actual selection gave a realized heritability of 0.65 (0.16). After major improvement in reduction of mortality due to beak-inflicted injuries in the first generation there has been little additional improvement of mortality (Muir, 1996) in later generations. It was, therefore, hypothesized that there was a major gene involved. The power of using group selection might be that under competitive circumstances the usual gene model for a given genotype should be extended to include not only the direct effects of its own genes, but also the associate contributions from other genotypes of the group. In optimizing productivity in competitive environments, one does not select for those individuals that perform the best, rather one selects for the group that produces best (Griffing, 1967). This is an elegant method which might be promising in reducing FP behavior or cannibalism in practice in a relatively short time period.

Selection for the trait FP using traditional methods is a black box (Muir, 1996; Kjaer et al., 2001). It is of importance to get more insight in the phenotype FP and the results of selection on the behavioral traits. Knowledge of the genes influencing behavior will improve the understanding of what actually is selected for and what the effects are on the animals: is the welfare of the bird actually improved or is the result of selection a bird which is no longer capable to respond to stimuli from its environment. The study presented in this thesis does not give an answer to that issue but is an initial set up to come closer to the understanding which genes and biological pathways play a role in the behavior of the chicken.

The QTL study presented in chapters 3 and 5 indicate that FP at young and adult age are regulated by different QTL. It was shown that this was also valid for other types of behavior (chapter 6). This is important because it indicates that measuring FP for a selection program should be performed in adult birds as long as there is no reliable predictor at young age for performing FP behavior at adult age. In addition, it was shown that QTL for gentle FP are different from QTL for severe FP in adult birds (chapter 3). Further efforts in fine-

mapping of these QTL will reveal a better insight in the genetic difference between the two types of FP. This can be of interest for the definition of FP and fine tuning of the phenotype definition in future selection programs. Fine mapping of the QTL can generate new genetic markers linked to gentle or severe FP. Because heritability of FP behavior is low (chapter 2) the use of genetic markers may improve selection programs (Dekkers and Hospital, 2002).

In conclusion: despite the difficulties in identification of genes involved in complex traits, genetics offers a possibility to reduce FP using traditional methods. In addition, with the research presented in this thesis, an important step has been made in unraveling the genetic aspects of feather pecking behavior and prospects of designing genetic markers for FP are feasible.

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Summary

Changes in consumers demand for more animal friendly production of food products has led to a change in the legislation on animal welfare in Europe. With regard to chicken housing, cage systems are prohibited by 2012. Chickens have to be kept by animal friendly, e.g., in free-range housing systems. Feather pecking (FP) occurs in cage systems as well as in free-range housing systems, however, in free-range housing systems this phenomenon is more difficult to control. Although environmental influences, such as light intensity, feed, litter availability and stress can have an important effect on the expression of FP behavior in a flock, there is clear evidence that genetic differences between chicken lines in the propensity to FP do exist. The aim of the study presented in this thesis is to unravel the genetics of feather pecking behavior in laying hens.

Feather pecking behavior

Feather pecking behavior was studied extensively in two commercial White Leghorn laying lines. These lines were denoted as high FP (HFP) and low FP (LFP) line and differed consistently in their propensity to feather peck. In addition, they showed physiological differences in their corticosterone response to manual restraint. To study the genetics of FP, a large F_2 intercross (630 hens) of the HFP and LFP was created. In a parallel study alternative methods to characterize the pecking behavior of chickens were validated. A social FP test was developed to characterize the birds from the F_2 population. In order to screen the whole chicken genome for the presence of QTL, 180 evenly spaced micro-satellite markers were typed on the F_0, F_1, and F_2 animals. All F_2 hens were scored for different types of pecking behavior (gentle FP, severe FP, aggressive pecking, toe pecking, and ground pecking) in a social FP test at young and adult age. In addition, the F_2 hens were also scored for being pecked. Birds were tested in groups of five hens in an open-field with wood shavings on the floor. The observer watched the birds for ten minutes. In **chapter 2** the heritabilities for pecking traits were estimated based on the social feather pecking test on 6 and on 30 weeks of age. At 6 weeks of age, the h^2 for gentle feather pecking is 0.12 ± 0.07 and the h^2 for ground pecking at 6 weeks of age is 0.13 ± 0.07. At 30 weeks of age, the h^2 for gentle feather pecking is 0.15 ± 0.08 and the h^2 for ground pecking is0.30 ± 0.20. In **chapter 3** a QTL analysis was performed on the data of the social FP test. Data were analyzed in two ways: a half-sib analysis and a line-cross analysis in order to identify QTL. At 6 weeks of age, a suggestive QTL was detected on GGA10 (53 cM) for gentle FP using the line-cross program. At 30 weeks of age a

suggestive QTL was detected on GGA2 (243 cM) for gentle FP using the half-sib program. Using the line-cross program, for gentle FP a suggestive QTL was detected on GGA1 (134 cM) and for severe FP a significant QTL was detected on GGA2 (196 cM) using the line-cross program. These results indicate that is possible to detect QTL for FP behavior, which may result in a genetic marker for FP behavior in the future. In addition, QTL detected on 6 weeks of age were distinct from the QTL detected on 30 weeks of age.

Furthermore, in **chapter 5** a QTL analysis was performed on the data from the same experiment but now for being pecked. At the age of 6 weeks a significant QTL was detected on GGA1 (356 cM) and two suggestive QTL were detected on GGA6 (4 cM) and GGA7 (114 cM) for receiving gentle pecks. At 30 weeks of age a suggestive QTL was detected on GGA5 (166 cM). In addition, at 30 weeks of age a suggestive QTL for receiving aggressive pecking was detected on GGA3 (231 cM) and for receiving toe pecking three suggestive QTL were detected on GGA1 (314 cM), GGA5 (77 cM), and GGA23 (8 cM). Overall the results on FP traits presented in **chapter 3** and **chapter 5** show that QTL found at young age do not coincide with QTL found for the same trait at adult age. Which is an indication that the behavior at young age is regulated by different genes than at adult age. Furthermore, QTL found for the different types of pecking traits and receiving pecking traits do not coincide as well. Indicating that whether a bird is pecking or is pecked is regulated by different genetic mechanisms. These observations were supported by a correlation study.

Corticosterone in relation to FP

An interesting feature of the HFP and LFP lines is that there is a physiological difference between these lines. The HFP line has a low corticosterone response, where the LFP line show a high corticosterone response to the manual restraint test, i.e., keep a bird on its side by hand for 8 minutes. Based on this observation, it was proposed that these laying lines fit in the coping style theory as defined for rodents. A coping style is defined as a coherent set of behavioral and physiological measures. There are two coping styles defined, a pro- active coping style characterized by an active behavioral response (fight/flight) and low corticosterone response; and a re-active coping style characterized by a passive behavioral response (conservation/withdrawal) and high corticostrone response. Birds from the HFP line may be characterized as pro-active and birds from the LFP line may be characterized as re-active copers. The hypothesis is that ‘FP represents a failure of

normal adaptive mechanisms to operate, and that glucocorticoids play a crucial role in this process'. The action of corticosterone on behavior act via the mineralocorticoid – glucocorticoid receptor balance. This makes the glucocorticoid receptor as well as the mineralocorticoid receptor interesting candidate genes.

In **chapter 3** a QTL analysis was performed on data obtained from the manual restraint test. Two suggestive QTL were detected on GGA15 (35 cM) using the line-cross analysis and on GGA18 (33 cM) using the half-sib analysis, respectively. These QTL do not coincide with the QTL for FP behavior, indicating that acute corticosterone levels in response to manual restraint is not a good predictor for FP behavior in adult birds. In **chapter 4** the glucocorticoid receptor was mapped to the chicken genome linkage map on GGA13. This study was extended by making a detailed comparative chicken-human genome between human chromosome HSA5 and chicken chromosome GGA13. The comparative map reveal intra-chromosomal rearrangements, which is consistent with the literature on comparative mapping between chicken and human. Based on its mapping location, the glucocorticoid receptor is no likely candidate gene for the stress response as well as for FP traits.

Open-field behavior

Using the HFP and the LFP lines, feather pecking has been associated with open-field response. In general, the open-field test has been used to study emotional reactivity and motivation in rodents as well as in poultry. The open-field is an square of 1.25m x 1.25m and is divided into 25 squares. The F_2 animals were tested individually at 5 weeks and 29 weeks of age. The activity of an animal can be scored by the number of squares crossed. General activity measures (sitting, standing, walking, number of steps, flying, and defecating) and vocalizations were recorded as well. In **chapter 2** heritabilities were estimated based on the open-field test for two different ages. In general, heritabilities were higher at young age than at adult age for both general activity measures as well as for vocalizations. In **chapter 6** a QTL analysis was performed on the data of the open-field test. Because the general activity measures were highly correlated, a factor analysis was performed to group the different traits in uncorrelated factors. Using the factor scores as a trait, a significant QTL was detected at 5 weeks of age on GGA4 (167 cM) and at 29 weeks of age a significant QTL on GGA4 (66 cM) was detected. It is not likely that these

QTL are the same, because the distance is more than 100cM apart, which consistent is with the results described in **chapter 3** and **chapter 5**.

In the general discussion (**chapter 7**) the main findings from chapters 2 to 6 are used to discuss five topics. The definition of the phenotype is of importance to have a clear measure of FP. The possible influences of the environment on behavior such as group effect are discussed. It is concluded that the social test is a reliable test to measure FP behavior. It was proposed that the coping style theory as described for rodents could be applied to chicken. Based on the results of this thesis it is clear that corticosterone is not a reliable predictor for FP behavior in adult hens. Based on the coping style theory the glucocorticoid receptor was proposed to be a strong candidate gene in for the regulation of FP behavior. Based on the comparative map in combination with the QTL mapped for corticosterone response and FP behavior it was concluded that based on the location on GGA13 the glucocorticoid receptor was not a good candidate gene. The QTL mapped for behavior in chicken generate the possibility to identify the underlying genes. This is, however, not very easy. Fine-mapping of the QTL of interest is feasible because the marker density can still be improved in the F_2 generation. In addition an F_4 generation is available which offers a great opportunity to search for linkage disequilibrium blocks. Nevertheless, there are some major concerns with the complexity of the interaction of genes underlying QTL. New technology such as interference RNA is discussed to unravel the genetics of complex traits, however, in chicken generating knockout or knockdown animals is still not possible. Despite the difficulties in identification of genes involved in complex traits, genetics offers a possibility to reduce FP using traditional methods. In addition, with the research presented in this thesis, an important step has been made in unraveling the genetic aspects of feather pecking behavior and prospects of designing genetic markers for FP are feasible.

Samenvatting

De maatschappij wordt zich steeds meer bewust van de omstandigheden waaronder producten zoals vlees en eieren geproduceerd worden. Als gevolg daarvan groeit de vraag naar diervriendelijker productiemethoden. Dit heeft geleid tot wettelijke maatregelen op Europees niveau. Voor legkippen betekent het dat individuele huisvestingssystemen verboden worden in 2012. In de toekomst zullen kippen in alternatieve huisvestingsvormen gehouden moeten worden, zoals verrijkte kooien, volièrestallen en systemen met uitloop naar buiten. Een groot probleem in de pluimveehouderij is verenpikken. Verenpikken komt voor in alle huisvestingsvormen, maar waar grotere groepen bij elkaar gehouden worden, is het moeilijker te beheersen. Het pikken en uittrekken van veren heeft een negatieve invloed op het welzijn van de kippen en resulteert in hogere voerkosten en sterfte. Er zijn verschillende soorten pikgedrag te onderscheiden, namelijk:

- Zacht verenpikken, waarbij een kip zich richt op een bepaalde plek van zijn slachtoffer en daarbij schade aan het verenkleed toebrengt.
- Hevig verenpikken, waarbij een kip hard op zijn slachtoffer inpikt en soms probeert de veren uit te trekken en zelfs op te eten. Dit leidt tot wondjes bij het slachtoffer en kan uiteindelijk resulteren in kannibalisme.
- Agressief pikken, waarbij een kip zich richt op de kop en/of nek van zijn slachtoffer.
- Kannibalisme, dat kan voortkomen uit hevig verenpikken en resulteert in de dood van het slachtoffer.

Het is bekend dat omgevingsfactoren zoals lichtsterkte, groepsgrootte of bezettingsgraad invloed hebben op het al dan niet voorkomen van verenpikgedrag. Wanneer gebruik gemaakt wordt van strooisel op de vloer en het verstrooien van het voer, zodat de dieren moeten scharrelen, vermindert dit de kans op verenpikken. Ook speelt stress een rol bij de initiatie van verenpikgedrag. Naast de omgevingsfactoren speelt genetische aanleg voor verenpikgedrag ook een rol.

In dit proefschrift wordt het promotieonderzoek beschreven van de afgelopen vier jaar. De doelstelling van dit onderzoek was uit te zoeken in hoe verre verenpikgedrag bij leghennen erfelijk is en welke genen daar een rol bij spelen. Dit is uitgezocht in twee commerciële Witte Leghorn lijnen. Deze lijnen zijn geselecteerd op eiproductie-kenmerken, zoals het aantal eieren, eigewicht en breuksterkte. Naast het verschil in eiproductie, vertonen deze lijnen een verschil in verenpikgedrag. Daarom worden ze de hoge verenpiklijn (HFP) en de lage verenpiklijn (LFP) genoemd. Ook verschillen de lijnen in openveldgedrag en corticosteronrespons wanneer de kippen aan stress blootgesteld worden. De dieren van de

HFP lijn vertonen een lage corticosteronrespons wanneer deze getest worden met de ‘manual restraint’ test. Bij de LFP is het omgekeerde het geval, namelijk de dieren van de LFP vertonen een hoge corticosteronrespons in de ‘manual restraint’ test. De ‘manual restraint’ test is een test waarbij de kip gedurende 8 minuten met de hand op haar zij gehouden wordt. De corticosteronrespons in de HFP en LFP lijn lijkt te passen in de zogenaamde ‘coping-style’ theorie die gebaseerd is op het gedrag van knaagdieren, zoals de muis en de rat. Hierbij wordt gesteld wordt dat pro-actieve dieren gekarakteriseerd kunnen worden als dieren die een ‘fight/flight’ reactie vertonen en daarbij een laag corticosteronniveau hebben. Omgekeerd, de re-actieve dieren kunnen gekarakteriseerd worden als dieren die een ‘conservative/withdrawal’ reactie vertonen en daarbij een hoog corticosteronniveau hebben.

De hypothese is dat corticosteronniveau in het bloed het gedrag reguleert waarmee een dier op zijn omgeving reageert. Het corticosteronniveau wordt gereguleerd door genen. Op basis van waarnemingen in knaagdieren zou de mineralocorticoidreceptor al dan niet in samenwerking met de glucocorticoidreceptor een belangrijke rol spelen in de regulering van de stressrespons en zo dus ook (in)direct het gedrag van het dier beïnvloeden.

Opzet van de testpopulatie

Voor de opzet van de test populatie is gekozen voor een zogenaamde F_2-populatie. Het idee achter deze opzet is dat allelen (verschijningsvormen van een gen) uitsplitsen in de opeen volgende generaties. Stel bijvoorbeeld dat de HFP als allelen AA heeft en dat de LFP als allelen aa heeft. Wanneer deze lijnen gekruist worden (AA x aa) dan heb je in de F_1-dieren de allelen Aa. Er wordt namelijk een A doorgegeven via de HFP lijn en een a via de LFP lijn. Als nu de F_1-dieren onderling met elkaar gekruist worden (Aa x Aa) krijg je een mengeling van allelen in de F_2-populatie, namelijk: AA, Aa en aa. Als de F_2-dieren gekarakteriseerd worden voor het pikgedrag, verwacht je zwart-wit gesproken drie vormen te vinden namelijk: hoog, middelmatig en laag. De opzet van de verenpiktestpopulatie ziet er als volgt uit: Er zijn 6 hanen van de HFP gekruist met 6 hennen van de LFP (1 op 1) en ook de omgekeerde kruising: 6 hennen van de HFP met 6 hanen van de LFP. Dit resulteerde in totaal in 120 F_1-dieren. Uit deze 120 F_1-dieren zijn 7 hanen gekruist met elk 4 hennen, hetgeen resulteerde in een F_2-populatie van 630 hennen.

Fenotyperen

Fenotyperen betekent een waarneming doen aan het uiterlijk van een dier.

Verenpiktest

Alle 630 dieren uit de F_2-populatie zijn gekarakteriseerd voor verenpikgedrag op jonge (6 weken) en op volwassen leeftijd (30 weken) met behulp van een test, waarbij verenpikken in groepsverband wordt bestudeerd. Groepjes van vijf dieren worden gedurende 10 minuten getest in een vierkante bak van 1.25m x 1.25m met zaagsel op de grond. Daarbij is gekeken naar zacht verenpikken, hevig verenpikken alsmede naar andere vormen van pikken zoals agressief pikken, grondpikken en teenpikken. Ook is er gekeken naar welke dieren gepikt werden. Deze test is gevalideerd in een parallel onderzoek (T. B. Rodenburg: Feather pecking and related behavioural characteristics in laying hens).

Open-field test

Op 5 weken en op 29 weken leeftijd is het gedrag van de F_2-dieren bekeken in een open-field test. Deze test wordt veelvuldig gebruikt als mate voor 'emotionaliteit' in knaagdieren. Bij de open-field test wordt een kip individueel in een open ruimte geplaatst. Op de grond zijn een 25 tal vakken getekend van elk 25 cm^2. Als maat voor activiteit worden een aantal gedragingen gemeten zoals de tijdsduur dat: een dier blijft zitten, blijft staan of de tijd tot de eerste alarm roep. Daarnaast zijn ook het aantal stappen, het aantal stressroepen, het aantal keren poepen en de frequentie van vliegen gemeten.

Manual-restraint test

Op 32 weken leeftijd zijn de F_2-dieren getest op hun stressrespons door middel van de zogenaamde 'manual restraint' test. Bij deze test wordt de kip gedurende 8 minuten handmatig op haar zij gehouden. Na deze 8 minuten wordt er een bloedmonster genomen, waarin de corticosteronconcentratie gemeten wordt. Corticosteron is een hormoon dat vrij komt bij stress en wordt veelvuldig gebruikt als een maat voor de mate van stress.

Genotyperen en analyse

Genotyperen betekent dieren karakteriseren op DNA-niveau, met andere woorden voor de erfelijke aanleg. De ontwikkelingen in de moleculaire genetica en de statistische methoden hebben het mogelijk gemaakt om genotype en fenotype aan elkaar te koppelen. DNA is opgebouwd uit 4 basen (A, T, C en G). Op het DNA komen stukken voor waar steeds twee basen herhaald worden, zoals $(TG)_n$. Er is variatie in het aantal keer dat een TG herhaald wordt, bijvoorbeeld $(TG)_{10}$ of $(TG)_{15}$. Deze variatie wordt ook wel aangeduid als allel en zijn

te gebruiken als DNA-merker. Met deze DNA-merkers is het mogelijk om de uitsplitsing van deze merkerallelen in de populatie te volgen. De ligging van deze merkers op een chromosoom zijn vastgesteld op de zogenaamde linkagekaarten. De afstanden tussen de merkers wordt gemeten in centiMorgan (cM). Dit is een maat voor hoeveel recombinatie er tussen twee merkers plaats vindt. Recombinatie is het aantal breuken dat plaatsvindt in het chromosoom bij de celdeling. Hoe groter de afstand tussen twee merkers, des te groter de kans op recombinatie.

Aan de hand van de linkagekaarten kun je de merkers ordenen, toevoegen aan een chromosoom en ook de volgorde ten opzichte van elkaar op het chromosoom bepalen. Op deze manier kun je de uitsplitsing van merkerallelen (AA, Aa, aa) over het totale genoom volgen. Voor deze studie zijn 180 DNA merkers gebruikt en op alle dieren in de populatie getest. Met behulp van statistische methoden is het mogelijk om nu het genotype en het fenotype met elkaar te associëren. Bijvoorbeeld als veel verenpikken altijd samengaat met AA en weinig verenpikken altijd samengaat met aa, dan is er sprake van associatie. Op deze manier wordt duidelijk welke plaats op een specifiek chromosoom een rol speelt bij verenpikgedrag. Dit wordt ook wel het quantitative trait locus (QTL) genoemd.

Resultaten

In hoofdstuk 2 is een schatting gemaakt hoe erfelijk verschillende soorten pikgedrag zijn op jonge en op volwassen leeftijd. Dit wordt uitgedrukt in een erfelijkheidsgraad (h^2). Deze h^2 geeft weer hoeveel procent van de totale variatie verklaard wordt door een erfelijke component. Het blijkt dat zacht verenpikken zowel op jonge als op volwassen leeftijd erfelijk is, maar dat de h^2 niet hoog is. Voor hevig verenpikken is de h^2 zowel voor jonge als volwassen leeftijd erg laag. De h^2 voor grondpikken is in jonge dieren lager dan op volwassen leeftijd. Voor openveldgedrag is de h^2 op jonge leeftijd hoger dan op volwassen leeftijd. In de praktijk zijn diverse kenmerken met een lage h^2 waarop met succes wordt gefokt, maar het vergt veel selectieinspanning.

In hoofdstuk 3 is een QTL analyse beschreven voor zacht verenpikken, hevig verenpikken, agressief pikken en teenpikken. Daarbij is voor zacht verenpikken op jonge leeftijd een suggestief QTL gedetecteerd op kippen chromosoom (GGA) 10 (53cM). En op volwassen leeftijd zijn er suggestieve QTL gedetecteerd op GGA1 (134 cM) en GGA2 (243 cM). Voor hevig verenpikken op volwassen leeftijd is er een significant QTL gedetecteerd op GGA2 (196 cM). Voor agressief pikken is er een chromosoom wijd significant QTL

gedetecteerd (GGA12 (33 cM)). Voor teenpikken zijn er geen QTL gedetecteerd. Voor corticosteronrespons zijn twee QTL gedetecteerd op GGA15 (35 cM) en GGA18 (33 cM). Uit dit hoofdstuk blijkt dat het mogelijk is dat er QTL gedetecteerd kunnen worden voor gedrag in de kip. Met name het QTL op GGA2 voor hevig verenpikken is interessant omdat deze vorm van pikken tot grote schade bij het slachtoffer leidt.

Gedrag is een moeilijk kenmerk om te meten. Uit gedragsstudies met genetisch identieke muizen blijkt bijvoorbeeld dat wanneer dezelfde gedragstest uitgevoerd wordt in verschillende laboratoria, de uitkomst van die test grote verschillen vertonen ondanks het feit dat alle protocollen hetzelfde zijn. Dat duidt erop dat omgevingsfactoren een belangrijke rol spelen op het gedrag. Dit kan ook van belang zijn, wanneer er gekeken wordt naar verenpikgedrag bij kippen. De verenpiktest blijkt een bruikbare test te zijn om te gebruiken voor het karakteriseren van dieren voor het pikgedrag. Met het oog op de invloed van de omgeving, zou het interessant zijn om na te gaan in hoeverre deze test bruikbaar is onder meer extreme omstandigheden, zoals in hokken zonder strooisel.

In hoofdstuk 4 is een gedetailleerde genenkaart gemaakt van kippenchromosoom GGA13. De volgorde van de genen op GGA13 is vergeleken met de volgorde van de genen op chromosoom 5 bij de mens (HSA5) en met de volgorde van de genen op chromosoom 11, 13 en 18 van de muis (Mmu11, Mmu13 en Mmu18). Het doel van een vergelijkende genenkaart is om een beter inzicht te krijgen in de volgorde van de genen bij de kip. Uit deze studie blijkt dat de volgorde van de genen bij de kip heel anders kunnen zijn dan de volgorde van de genen bij de mens. Dergelijke gedetailleerde kennis van de volgorde van genen is van belang voor het zoeken van kandidaatgenen. Het glucocorticoidreceptorgen is een kandidaatgen voor gedrag en is nu geplaatst op de genenkaart bij de kip op GGA13. De locatie van het glucocorticoidreceptorgen komt niet overeen met de QTL regio's die gevonden zijn voor verenpikgedrag en de stressrepons. Dit maakt dit gen een minder waarschijnlijk kandidaatgen voor verenpikgedrag en stressrespons bij de kip.

Op basis van de 'coping-style' theorie gaat een laag corticosteronniveau samen met actieve dieren en omgekeerd, een hoog corticosteronniveau gaat samen met passieve dieren. Op lijn niveau, dat wil zeggen in de ouderlijnen (LFP en HFP) vind je dat dieren van de LFP lijn een hoog corticosteronniveau vertonen wanneer deze getest worden in de 'manual restraint test'. Het omgekeerde geldt voor dieren uit de HFP lijn. De QTL die gevonden worden in de F_2-populatie voor de stressrespons komen niet overeen met de QTL die gevonden worden voor verenpikgedrag, hetgeen erop zou kunnen duiden dat corticosteron

geen voorspellende waarde heeft voor verenpikgedrag. Op basis van de literatuur echter, lijkt het er wel op dat verenpikken geïnitieerd wordt door stress. Om een beter inzicht te krijgen of corticosteronniveau een voorspellende waarde heeft voor het vertonen van verenpikgedrag, zou er nog een correlatiestudie uitgevoerd moeten worden. Daarbij kan onderscheid gemaakt worden tussen de fenotypische correlatie en de genetische correlatie. De fenotypische correlatie geeft de samenhang tussen twee kenmerken weer op fenotypisch niveau. De genetische correlatie geeft weer in hoeverre twee kenmerken genetisch overeenkomen. Dit geeft inzicht in het feit of twee kenmerken (bijvoorbeeld corticosteronrespons en verenpikken) aangestuurd worden door dezelfde genen.

In hoofdstuk 5 is een QTL analyse beschreven voor de verschillende vormen van gepikt worden. Op jonge leeftijd is een significant QTL gevonden op GGA1 (356 cM) en drie suggestieve QTL regio's. Te weten, op GGA2 (269 cM), GGA6 (4 cM) en GGA7 (114 cM). Op volwassen leeftijd wordt er een suggestief QTL gevonden op GGA5 (166 cM). Tevens zijn er drie suggestieve QTL gevonden voor het op de tenen gepikt worden op respectievelijk GGA1 (314 cM), GGA5 (77 cM) en GGA23 (8 cM).

In hoofdstuk 6 is een QTL-analyse beschreven voor open veld gedrag op jonge en op volwassen leeftijd. Omdat de verschillende gedragselementen sterk gecorreleerd zijn, is er een factoranalyse uitgevoerd om de belangrijkste gedragselementen (b.v. lopen, poepen, vliegen) van elkaar te maken en te kijken welke waarnemingen horen bij die gedragselementen. Dit resulteert in de zogenaamde factorscores, waarbij verschillende gedragselementen die sterk met elkaar gecorreleerd zijn in een factor ingewogen zijn. Deze factor wordt factor 'angst' genoemd, omdat de gedragselementen die te maken hebben met angst sterk op deze factor laden. Op jonge leeftijd is een significant QTL gevonden op GGA4 (167 cM) en een suggestief QTL op GGA2 (213 cM). Op volwassen leeftijd wordt een significant QTL gevonden op GGA4 (66 cM) en een suggestief QTL op GGA1 (106 cM). De afstand tussen de QTL op GGA4 is zo groot dat het niet waarschijnlijk is dat deze dezelfde QTL zijn. De resultaten komen overeen met wat ook gevonden is voor verenpik gedrag en gepikt worden, namelijk dat QTL op jonge leeftijd niet overeen komen met QTL op volwassen leeftijd. Dat duidt erop dat gedrag op jonge leeftijd aangestuurd wordt door andere genen dan gedrag op volwassen leeftijd.

Verder onderzoek en conclusie

Het vinden van een QTL is slechts een eerste stap in de zoektocht naar de genen voor verenpikgedrag. Een vervolgstap die gemaakt zou moeten worden om de causale genen te vinden is bijvoorbeeld om meer DNA-merkers te gebruiken in de F_2-populatie in combinatie met extra vervolg generaties, om zo het betrouwbaarheidsinterval van het QTL te verkleinen. Vrij recent onderzoek in mens en muis toont aan dat er in het DNA gebieden zijn waar weinig tot geen recombinatie plaatsvindt. Dit wordt aangeduid met 'haplotype' blokken. Dit zijn de kleinst mogelijke gebieden die aangetoond kunnen worden met behulp van DNA-merkers die dicht bij elkaar liggen. Op dit moment echter, zijn er nog te weinig merkers beschikbaar om een goede schatting te maken van deze 'haplotype' blokken. Het is daarom het belangrijk in de vervolg fase nieuwe DNA-merkers te ontwikkelen. Wanneer de 'haplotype' blokken gedefinieerd zijn, komt er een nieuw probleem om de hoek kijken, namelijk dat de effecten van de genen die in dat 'haplotype' blok liggen, niet meer onderscheiden kunnen worden, omdat er geen recombinatie tussen deze genen plaatsvindt. Om de invloed van de individuele genen te kunnen bestuderen zijn er nieuwe technieken nodig, zoals micro-arrays, om gen-expressie te kunnen meten. Een andere mogelijkheid zou kunnen zijn om analoog aan de muismodellen, kipspecifieke deletie-mutanten te maken. Ondanks verscheidene pogingen, is dit echter tot nu toe nog niet gelukt. Een andere mogelijkheid die bediscussieerd wordt, is het gebruiken van interference RNA (iRNA). Bij deze techniek spuit je complementair RNA (= iRNA) van je kandidaatgen in. Dit iRNA hecht zich aan het mRNA van het kandidaatgen waardoor het gen niet tot expressie komt. Op deze manier kunnen de individuele geneffecten bestudeerd worden. Ook hier geldt dat de methode nog niet voorhanden is in de kip. Ontwikkeling van dergelijke technieken voor de kip is essentieel voor de verdere ontrafeling van de genen die een rol spelen bij kwantitatieve kenmerken.

Ondanks de moeilijkheden bij de identificatie van genen die een rol spelen in kwantatieve kenmerken, kunnen we concluderen dat genetica een mogelijkheid biedt om het verenpik probleem aan te pakken met behulp van conventionele fokkerij. Middels het onderzoek dat beschreven wordt in dit proefschrift is een belangrijke stap gezet in de ontrafeling van de genetische achtergrond van verenpikgedrag in leghennen.

List of abbreviations

ABR	National Institute of Agrobiological Resources, Kannandai, Japan
ADL	Avian Disease and Oncology Laboratory, Michigan State University, East Lancing, USA
BAC	Bacterial Artificial Chromosome
cM	centi Morgan
CORT	Corticosterone
EST	Expressed Sequence Tag
FISH	Fluorescent *in situ* Hybridization
FP	Feather Pecking
GGA	Gallus gallus (chicken) chromosome
h^2	heritability
HFP	High Feather Pecking line
HSA	Homo sapiens (human) chromosome
LFP	Low Feather Pecking line
LEI	University of Leicester, Leicester, UK
MCW	Microsatellite Chicken Wageningen
MMU	Mus musculus (mouse) chromosome
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Locus
SNP	Single Nucleotide Polymorphism
STS	Sequence Tag Site
WAU	Wageningen University, the Netherlands

List of publications

Related to this thesis

Buitenhuis, A. J., Crooijmans, R. P. M. A., Bruijnesteijn van Coppenraet, E. S., Veenendaal, A., Groenen, M. A. M. and J. J. van der Poel. 2002. Improvement of the comparative map of chicken chromosome 13. Anim. Genet. 33: 249-254.

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Nawoord

Het heeft iets meer dan vier jaar geduurd, maar nu is het ei dan ook gelegd. Zonder de hulp van velen was het zeker niet gelukt. Graag wil ik een paar mensen met name noemen.

Jan, ik weet nog goed dat je mij deze baan toe zegde. Wat was ik blij. Bedankt voor de fijne samenwerking en je positieve instelling. Je bent altijd enthousiast en als het soms wat tegen zat, wist je steeds weer de zonnige zijde te laten zien. Daarnaast heb je me vrijgelaten in planning en in doen en laten. Ook heb je altijd tijd voor een praatje over de dingen van alledag of een stevige discussie. Dat heb ik erg gewaardeerd!

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Lopen een Fokker en een Etholoog samen over een weg, zegt de Fokker tegen de Etholoog: “Zou jij even 630 kippen willen fenotyperen?” Waarop de Etholoog zegt: “……!?!?”. Bas, je hebt (dankzij de fokkers in het project) een groot deel van je tijd doorgebracht in de stal. Gelukkig is het vele werk allemaal niet voor niets geweest en mag het resultaat er zijn. Bedankt voor de relaxte samenwerking!

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Daarnaast wil ik ook Yvonne, Mechiel, Ton, Bernd en Harry bedanken voor de discussies tijdens de geregelde bijeenkomsten.

Maria, our projects were in the same stage with regard to data collection and QTL-mapping. For me, it was very helpful to have someone facing the same type of problems. I really appreciated our discussions and chats! You were always ready to give a hand when needed. Thanks a lot for all the help in genotyping, stable work, QTL-mapping etc., etc.

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Als je DNA nodig hebt, moet je eerst bloed hebben. Mike, de snelheid waarmee jij bloed prikt kan ik nog steeds niet evenaren. Daarnaast was je ook altijd bereid om mee te werken aan andere ‘stal acties’ zoals de ‘manual restraint test’. Bedankt voor je hulp.

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Danyel, als kamergenoot moest je het met mij doen de afgelopen vier jaar ☺? ☹?. Bedankt voor je interesse en gezelligheid.

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Curriculum Vitae

Albert Johannes (Bart) Buitenhuis werd op 3 oktober 1973 te Rotterdam geboren. In 1991 behaalde hij het HAVO diploma aan de St. Andreas Scholengemeenschap te Zevenaar. Het VWO diploma behaalde hij aan het Liemers College te Zevenaar. Zijn opleiding aan de Christelijk Agrarische Hogeschool te Dronten met als specialisatie Veehouderij en Biotechnologie heeft hij afgerond in 1997. In datzelfde jaar begon hij zijn MSc-opleiding Animal Sciences aan de toenmalige Landbouwuniversiteit te Wageningen. Zijn eerste afstudeervak heeft hij gedaan bij de leerstoelgroep Fokkerij & Genetica. Hier heeft hij gewerkt aan de optimalisatie van de screening van de kippen-BAC-library. Zijn tweede afstudeervak van 4 maanden voor dezelfde vakgroep werd uitgevoerd aan de Iowa State University, Ames, Iowa, USA. Tijdens dit afstudeervak is gekeken naar associatie van kandidaat genen met immuun respons kinetiek kenmerken tegen SRBC en BA in de kip. In 1999 is hij afgestudeerd met als specialisatie Fokkerij-moleculair, waarna hij in maart 1999 is begonnen als Onderzoeker In Opleiding (OIO) bij de leerstoelgroep Fokkerij & Genetica aan het in dit proefschrift beschreven promotieonderzoek.

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