Genetic Dissection of Growth and Fatness

Using Divergent Intercrosses in Chickens

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Abstract

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Most phenotypes in human and animals have a multifactorial background, e.g. they depend on many different genes and environmental factors may play a prominent role. Geneticists have long been concerned with identifying key genes responsible for variation in multifactorial traits, such as obesity and diabetes in humans and production traits in animals. In this thesis I have used two different intercrosses to map Quantitative Trait Loci (QTL) for growth and production traits in chicken.

The first part of the thesis is based on an intercross between White Leghorn and the ancestor of the modern domesticated chicken, the red junglefowl. A total of 13 QTLs reached genome-wide significance and the four major QTLs explain around 80% of the phenotypic variance between the parental populations in males, indicating that a few QTLs have had a large influence on the enhanced growth rates in domesticated chickens.

The second intercross is between two chicken lines divergently selected for body weight at 56 days of age. The selection response has been remarkable and after 42 generations of selection the lines differ almost nine-fold in weight. QTL studies revealed 13 QTLs for growth and each QTL explains a small part of the phenotypic variance within the F_2 generation. Altogether, the 13 QTLs explain a smaller part of the population variance compared to the red junglefowl x White Leghorn intercross. For each QTL, the allele from the high line was associated with enhanced growth.

Finally, the gene coding for *melanocortin receptor 3* (*MC3R*) was evaluated as a positional candidate gene for an early growth QTL on chromosome 20 in chicken. The analysis showed that the high and low lines are fixed for different *MC3R* alleles, an observation that strengthens *MC3R* as a positional candidate gene. Expression analysis revealed a significant differential expression with higher expression in the low line at hatch. Further analyses indicated that this differential expression was primarily due to trans-acting factor(s). The two large QTL studies presented in the thesis has potential to result in identification of causative trait nucleotides for production traits in chickens as well as interesting candidate target genes for human metabolic disorders.

Keywords: chicken, linkage map, Quantitative Trait Loci, growth, egg production, appetite regulation, body composition, metabolic traits, *MC3R*.

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Contents

List of papers

The thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Jacobsson, L., Park, H.B., Wahlberg, P., Jiang, S., Siegel, P.B. & Andersson, L. 2004. Assignment of fourteen microsatellite markers to the chicken linkage map. *Poultry science* 83:1825-31.

II. Kerje, S., Carlborg, Ö., Jacobsson, L., Schütz, K., Hartmann, C., Jensen, P. & Andersson, L. 2003. The twofold difference in adult size between the red junglefowl and White Leghorn chickens is largely explained by a limited number of QTLs. *Animal genetics* 34:264-274.

III. Jacobsson, L., Park, H.B., Wahlberg, P., Fredriksson, R., Pérez-Enciso, M., Siegel, P.B. & Andersson, L. Many QTLs, ech with a small effect, explain a large difference in growth between two selection lines in chicken. *Manuscript*.

IV. Park, H.B., Jacobsson, L., Wahlberg, P., Siegel, P.B. & Andersson, L. QTL analysis of body composition and metabolic traits in an intercross between chicken lines divergently selected for growth. *Manuscript.*

V. Jacobsson, L., Wahlberg, P., Ka, S., Park, H.B., Jiang, S., Denbow, D.M., Siegel, P.B., Hallböök, F. & Andersson, L. Evaluation of *MC3R* as a positional candidate gene for a growth QTL in chicken. *Manuscript*.

Introduction

Complex traits, such as height or weight, depend both on genetic as well as environmental factors. The genetic component is difficult to elucidate as an unknown number of loci have different effects on the trait. In this thesis, I have utilised the enormous diversity among domestic chickens (Andersson, 2001) to dissect the genetics behind complex traits.

Domestication

Domestication is the process, by which humans have altered phenotypic traits in plants and animals to better fit their needs. This has been executed by conscious control of reproduction and has resulted in the variety of domestic animal breeds that we have today. The dog was the first animal to be domesticated as early as ~15,000 years ago, and it was followed by domestication of cattle, pig, goat and sheep around 9,000-11,000 years ago according to archaeological remains. The domestication events are beginning to be unravelled through molecular studies for several domestic species (Bruford, Bradley & Luikart, 2003) and future analyses of the breeds of today will shed further light on the domestication process.

Plants and animals were domesticated when the number of large prey decreased in combination with climate unpredictability caused the hunter-gatherer people to begin to form permanent farming settlements. Initially, the farmers had a worse nutritional situation, more work and were subject to more diseases than the hunter-gatherers, but eventually the advantages of permanent settlements and domestication of plant and animal species made the farmer lifestyle superior to hunter-gatherers and the culture along with the domesticated species spread across the world (reviewed by Diamond, 2002).

The earliest sign of domesticated chickens has been found in Neolithic sites along the Yellow River in northeast China. Some of the sites were at least 7,500 years old (West & Zhou, 1988). In ancient Egypt the first traces of chickens are from the XIX Dynasty (1292-1185 B.C.), based on a black ink drawing of a chicken found by Howard Carter in 1923. The primary purpose of the early domesticated chicken is unclear, but they are believed to have been used in religious ceremonies and for cockfighting.

Studies of mitochondrial DNA indicate that the red junglefowl (*G. gallus*) is the ancestor of all domesticated chickens (Fumihito *et al.*, 1994 and 1996), a fact that was already proposed by Darwin in 1868. Darwin summarized results from mating experiments with chickens and found that the red junglefowl was the only one of the

four wild Asian chickens (red, green, grey and ceylon junglefowl) that could produce fertile offspring with domesticated birds (Darwin, 1868).

Model animals

Animals have long been used as a tool to study basic biology and for understanding human diseases. Animals provide an excellent opportunity to study disease in controlled experiments. There are animal model systems for diverse human diseases such as obesity and Alzheimer's disease in mice (German & Eisch, 2004; Zhang *et al*., 1994), diabetes mellitus in rat (Janssen *et al*., 2004), rheumatoid arthritis in dogs (Carter, Barnes & Gilmore, 1999) and epilepsy in several species (reviewed by Fisher, 1989).

In animals, diseases can be studied in controlled environment compared to human studies, where the patients may have different backgrounds and life conditions that may influence the disease phenotype. Animals also generally have a shorter generation time, which facilitates studies of lifetime disease effects and inheritance patterns. In order to dissect the genetics of complex disease traits it may prove valuable to genetically manipulate individual components of a certain pathway or process. This is, if ethical, possible to do in animals.

The human genome sequence was published in 2001 (Lander *et al*., 2001) and started discussions regarding the need for animal models in the future. Some meant that the human sequence would render animal models redundant for studies of human disease in the near future. However, the complexity of the human genome and the difficulty in controlling environmental and social factors in human studies, as well as the long generation time will doubtlessly favour studies of complex diseases in model organisms.

The drawback with animal models is the fact that the studied trait may resemble a human disease but prove to be regulated in a different pathway or turn out to be different from the human version in other aspects. Then, finding the key mutation will not aid in developing a drug, but may help to figure out possible pathways involved in the disease.

Farm animal models

Darwin foresaw the potential in using farm animals for studies to understand the process of evolution. In "The Origin of Species" he devotes the first chapter to discuss "Variation under Domestication", where he uses the pigeon as an example for discussions around how selection affects a species during domestication (Darwin, 1859). Farm animals have been selected for optimised production, which has resulted in specialisation for different purposes, also within a

species. In cattle, two subdivisions of breeds have been developed for milk and beef production respectively. Milk producing cattle are optimized to generate maximal amounts of milk per feed intake, a process that involves for example lipid metabolism for milk fat content. Beef cattle metabolism is, however, focused on lean muscle mass. Pigs were selected for muscle mass and fatness up to the mid 1950-ies when consumers started to demand lean meat. Since then the lean muscle mass content have increased yearly in pigs. These specializations of breeds to meet certain consumer demands can be utilised in studies of fat and muscle metabolism as well as growth and resource allocation. Other traits, such as behaviour, resistance to disease, and skin and meat pigmentation have also been under selection.

Chicken as model animal

Chickens have traditionally been used for studies in developmental biology (Stern, 2005). The chicken egg provides an interesting and relatively easily manipulated *in vivo* system that developmental biologists have used for example to understand limb formation (Mariani & Martin, 2003). Gene constructs can be introduced into the egg through retroviral methods and recently RNAi methods have successfully been used to block transcription (Pekarik *et al*., 2003).

Chickens can serve as good models for several reasons. They are fairly easy and relatively cheap to maintain in larger numbers, they produce comparatively large numbers of offspring and many interesting characteristics are easy to score (body weight, plumage colour, body composition, metabolic and immunological traits, etc.). This, in addition to a short generation time and a high recombination rate render chickens suitable as models for genetic studies (Burt & Pourquie, 2003).

Great phenotypic diversity has developed within chickens since the evolution from their common ancestor, the red junglefowl. Intense selection has resulted in specialized egg layer and broiler chicken lines used for production. A tremendous variety of plumage colour, feather texture, comb forms as well as size are present among domesticated chicken lines. Many of these traits have been mapped to linkage groups and for some of them, for example the plumage colour loci *Extended black* (Kerje *et al*., 2003) and *Dominant White* (Kerje *et al.*, 2004), the causative genes and mutations have been identified, but for others the genetic background is poorly understood and awaits unravelling by determined geneticists.

Many mutant chicken strains exist including the OS-chicken line for autoimmune thyroiditis (summarized in Vasicek *et al*., 2001), lines for retinal degeneration (Semple-Rowland, 1998), the Smyth lines for vitiligo (Bowers, 1992) and the UCD-200 and 206-lines for

scleroderma (Sgonc *et al.*, 1995). Lately, the loss of interesting chicken strains, due to budget cuts at many universities and other facilities, has been brought up for discussion (Miller, 2004; Fulton & Delany, 2003). The scleroderma lines mentioned above is only one example of many lines that have been terminated. This is in contrast to the mouse genetics community, where mutant mice strains are being well preserved for future studies. A complicating factor for preservation of unique chicken strains is that the egg and sperm do not freeze well in contrast to mice where embryos can be stored by freezing. However, there are still many available chicken lines to be used for genetic studies of production traits and diseases.

Egg laying chickens are interesting because they provide an exceptional model for calcium and fat metabolism. In order to produce as much as one egg per day during a long period of time, the chicken need to take up calcium efficiently and store it in bone. Also fat is needed for egg production and traditionally the best egg layers were said to be just "bone and fat".

In the recently published chicken genome sequence paper comparisons were made between conserved non-coding regions in chickens, humans and rodents. It was found that only 30-40% of all non-coding regions that are conserved between humans and rodents are also conserved between humans and chickens. Elements involved in development, metabolism and muscle structural components are overrepresented among the conserved non-coding regions (Hillier *et al*., 2004). These data provide further support for chicken as a model for development, muscle structure and metabolism.

Chicken lines as model for growth and fatness

The genetics behind complex traits may be studied by intercrossing breeds with distinct phenotypic differences (briefly discussed in Methods). We have generated two such intercrosses; the first between red junglefowl and White Leghorn and the second between two lines divergently selected for body weight at eight weeks of age. Both intercrosses facilitate mapping of loci involved in growth and fatness-related traits. I will here provide background information for the founder lines of these intercrosses.

Red junglefowl

There are four types of junglefowl belonging to the *Gallus* genus; grey junglefowl (*G. sonnerati*), green junglefowl (*G. varius*), ceylon junglefowl (*G. lafyettei*) and red junglefowl (*G. gallus*). All four types originate from Asia, where they are still found in the wild. The grey, ceylon and red junglefowls prefer a forest and forest clearing habitat, whereas the green prefers the seashore (Crawford, 1990).

Red junglefowl (Figure 1), is the ancestor of all domesticated chickens. It still exists in the wild in Asia, although voices have been raised that the purity of these birds are doubtful and that intercrossing with domestic chickens have been more or less extensive. A number of red junglefowl populations are kept in zoos, university farms, as well as in some private facilities around the world. These populations stem from imported birds from various regions in Asia and they have been kept in captivity for various numbers of generations. The birds are selected for a "wild" phenotype in order to purge contaminating genetic material due to intercrosses with domestic chickens. Examples of criteria for selection are, for example horizontal tail-feathers in males and small comb in the females. It is hard to predict whether these red junglefowl populations have been intercrossed with domestic birds and whether their captive environment has resulted in any domestication event.

Figure 1. The red junglefowl (left) was intercrossed with White Leghorn (right) to generate a large F_2 mapping populatiuon. (Foto:Johanna Väisänen)

Red junglefowl differs from domestic chickens in a number of traits. For example all red junglefowl have virtually the same plumage phenotype whereas many different patterns and colours are present among domesticated chickens (Andersson, 2001). This is due to high natural selection pressure on the "wild" plumage in red junglefowl and to selection for different plumage colours in domestic chickens. The red junglefowl is a seasonal egg layer and both the female and male plumage on the neck changes after the breeding season (eclips). The colour of the eggs is white to rosy cream (Delacour, 1977). A male red junglefowl weights between 800-1360 grams and the female approximately half of that (Crawford, 1990).

White Leghorn

The Leghorn chicken breed is generally believed to originate from the city of Leghorn in Italy (American Poultry Association, 1947). The breed spread by boat to for example Great Britain where many of the different colour variants within the Leghorn breed were developed. The breed is known as extraordinary egg-layers and the commercial lines lay more than 300 eggs per year. The female broodiness (desire to incubate eggs) has been selected against and is now virtually eliminated from the breed. They reach sexual maturity early, which is an advantage in poultry production (egg-production). The White Leghorn produces pure white eggs and it is the most commonly used chicken breed for white egg production today.

The White Leghorn used in this study is from line 13 (Figure 1B), which has been kept at the Swedish University of Agricultural Sciences (SLU) until recently, when it was sold to a private farm. However, one population is kept at SLU in Skara. Line 13 was started in 1987 and has been selected for body weight, feed consumption, egg number, egg weight and change of egg size over age.

High and Low weight selection lines

The high and low weight selection lines (Figure 2) used in this study have been generated by Prof. Paul B. Siegel at the Virginia Polytechnic Institute and State University (USA). Seven inbred White Plymouth Rock chicken lines were intercrossed and two lines were established from this base population by selection for high and low body weight at 56 days of age. The selection experiment started in 1957 and one generation has been produced each year since then with a new hatch the 1st Tuesday in March. To minimize inbreeding the population has been kept in sufficient numbers. The population size was 8 males and 48 female for the first four generations, 12 and 48 from generation 5 (G_5) through G_{25}, and 14 and 56 from G_{25} onwards. The two divergent chicken lines were kept in the same facility under identical conditions and on the same feed for all generations. The alterations made during the experiment were vaccination for Marek's disease beginning in G_{17} and feed restriction from 56 days of age in the high line from G_{18}. Feed restriction was to avoid severe metabolic disorders within the high line because of excess body weight.

Selection for high and low body weight at 56 days of age has resulted in a remarkable selection response (Figure 2) and correlated responses in other traits including appetite, body composition, metabolic, reproductive and immune response traits. More than 100 papers have been published on these lines and I will here summarize some interesting findings related to our study. Informative review papers have been published by Liu, Dunnington & Siegel (1994,

1995a, b) and Dunnington & Siegel (1996). A review of feed intake regulation in birds with emphasis on these selection lines were published by Denbow (1999). In this summary I will provide references for key original papers, all other references are found in the recommended reviews. Table 1 summarises the correlated responses discussed here.

Figure 2. Response to selection for high and low 56-day weight. The selection started in 1957 and since then one generation has been produced every year. In the photo both the low and high weight line birds are 56 days of age (selection age), where they show almost a nine-fold weight difference. (Foto: Dr. E.A. Dunnington)

Appetite differences between the lines were noticeable after generation five and it has been shown that the two lines eat similar amount of feed per meal but the high line birds have more meals per day compared to birds from the low line (Barbato *et al*., 1980). Furthermore, feed intake was increased in the low line when plasma from feed-deprived high line birds was injected into their blood. These data suggest there is a factor in the plasma of high line birds that increases feed intake. The treatment had no significant effect on feed intake in the high line. Pair-feeding experiments showed that the high line is more efficient in feed conversion, e.g. the high line gain more weight on the same amount of feed than the low line (Barbato, Siegel & Cherry, 1983). Moreover, the feed efficiency in high line birds has been associated with thermoregulation, rate of feed passage and intestinal glucose absorption. Recently, Kuo *et al*. (2005) showed that administration of leptin increases feed intake in the low line, but not in the high line. The gene for leptin has not been identified in chicken, but the leptin receptor is present and apparently has a similar function as in mammals. Calbotta *et al.* (1983, 1985) showed that low line chickens have a higher lipogenic

and lipolytic capacity than high line birds, which suggest an important role of lipolysis in fat deposition.

Table 1. Phenotypic differences between the high and low weight selection lines. A plus (+) indicates higher values and a minus (-) indicates lower values.

Trait	High weight line	Low weight line
Feed (g) per meal	0	0
Meals per day	+	-
Feed intake after injection of plasma from feed-deprived High line chickens	0	+
Feed conversion	+	-
Feeding behaviour	Hyperphagic	Hypophagic / anorexic
Feed intake after leptin administration	0	+
Feed intake after lesion of hypothalamic brain region	0	+
Lipogenesis / lipolysis	-	+
Body fat	+	-
Muscle cells (no)	+	-
Muscle cell size	+	-
Breast muscle weight	+	-
Leg weight	+	-
Intestine size	-	+
Gizzard weight	-	+
Brain size corrected for body weight	-	+
Plasma conc. of glucose	+	-
lipid	+	-
proteins	+	-
IGF-I[(1)]	+	-
Number of eggs[(2)]	+	-
Egg weight	+	-
Embryo survival	-	+
Ab[(3)] response after SRBC[(4)] immunization	-	+
Persistency of Ab[(3)] response	-	+

0 : No change
[(1)] Insulin-like growth factor I
[(2)] High weight birds were feed restricted
[(3)] Antibody
[(4)] Sheep Red Blood Cells

Feeding behaviour was studied by O'Sullivan, Dunnington & Siegel (1992) and they documented hyperphagic behaviour in the high line and hypophagic in the low line. To investigate whether the eating behaviour of high line chickens could encourage low line chickens to feed more, birds from both lines were raised and fed together. Surprisingly, the high line chickens were consuming more when raised with low line chickens, whereas the low line chickens showed no difference in feed intake. Electrolytic lesion of the ventro-medial hypothalamus in chickens of the low line resulted in obesity but had no effect in chickens from the high line (Burkhart *et al.*, 1983), indicating that a factor in or from the hypothalamus of low line chickens inhibit feed intake and/or conversion, and that the high line

chickens lack this feed intake regulator or are insensitive to the presence of it.

The selection for low body weight has resulted in a condition resembling human anorexia. The anorexic trait in the low line is characterized by a number of birds dying soon after hatch and others failing to reach sexual maturity (egg production). Since the anorexic behaviour of the low line was first noticed in G_{25} and G_{26}, 25-50% of the low line birds fail to reach sexual maturity in each generation. These birds can, however, be brought into egg production by force feeding (Zelenka *et al*., 1988). In addition, 5-20% of newly hatched low line chicks die within the first couple of weeks because they never seem to start feeding. These birds have no food in the gut at the time of death, which strongly indicate they do not feed. Chickens survive for around one week without feeding because they survive on nutrients from the yolk sac.

Body composition studies show that high line chickens have higher body fat ratio, more and larger muscle cells, heavier breast muscles and legs and smaller intestine than low line chickens. The low line chickens, however, have heavier gizzards and feathers and a larger brain per gram body weight.

High line chickens have higher plasma concentration of glucose, lipids, insulin-like growth factor I (IGF-I), and proteins compared to low line birds. Furthermore, low line chickens clear glucose from blood more efficiently than high line chickens, possibly indicating insulin resistance in the high line. High line birds produce more eggs than the low line, but many of them are defective and multiple yolks are more common in the high weight line than in the low weight line.

The lines show different immune response to sheep red blood cells (Liu *et al*., 1995a). The low line has a more persistent immune response to sheep red blood cells (SRBC) than the high line and antibody titres are higher in the low line than in the high line.

Chicken genomics

The chicken genome

The chicken karyotype constitutes 38 autosomes and two sex chromosomes (Z and W). The chromosomes differ in size and are commonly divided into macro- and microchromosomes, although the genome sequence consortium suggests three groups, macro-, intermediate and microchromosomes.

Unlike mammalian sex chromosomes, it is the female chicken that is heterogametic (ZW) and the male homogametic (ZZ). Females are also heterogametic in butterflies and some fish species. The sex-determination genes have not yet been identified; it is unclear whether it is a sex determination gene or simply a dosage effect of the Z chromosome that determines sex (Schartl, 2004).

Genome sequence

The chicken genome sequence assembly was released in public databases in February 2004 and published in December the same year (Hillier *et al.*, 2004). A single female red junglefowl (UCD001) from University of California (Davis, USA) was sequenced. The published sequence provides a 6.6 X coverage and span 1.06 Gbp, resulting in a three-fold difference in size compared to the human genome (~3.0 Gbp).

Chromosome size is negatively correlated with recombination rate, GC-content and gene density confirming results from previous studies (Brown *et al*., 2003; Schmid *et al*., 2000; Smith *et al*., 2000). Also CpG-content is negatively correlated with chromosome size. Repeat density is, however, positively correlated with chromosome size. The difference in chromosome length is largely influenced by variations in intron lengths. Furthermore, 9% of the chicken genome consists of interspersed repeats compared to 40-50% in the human genome. The chicken genome consists of an estimated 20,000 to 30,000 protein coding genes, 80-90% of which was found in chicken EST databases. Around 60% of these coding genes have human orthologs and 72% of those are also conserved in the puffer fish (*Fugu rubripes*, Aparicio *et al.*, 2002).

The sequenced red junglefowl female came from the inbred UCD001 line which was described by Abplanalp in 1992. The population was started in 1925 has been kept inbred since then. The generation interval has been about 9-10 months, resulting in almost 100 generations of inbreeding. The inbred line was chosen because heterozygous loci would cause difficulties in sequence assembly. Since the female is the heterogametic sex in birds, sequencing a female bird would give sequence information for both sex chromosomes, although with decreased coverage compared to autosomes. The

UCD001 line was also one of the parents in the East Lansing mapping population (Crittenden *et al.,* 1993). The population is one of the three most used populations for mapping of molecular markers and construction of linkage maps in chicken.

In addition to the sequenced red junglefowl, another three chickens representing different domesticated breeds were sequenced for 25% coverage (Wong *et al*., 2004). The sequenced lines were broiler, chinese Silkie and one female layer from Line 13 at SLU. The Line 13 has been intercrossed with red junglefowl to generate a QTL mapping population, results from which is presented in this thesis. Comparisons of SNPs within and between the domesticated chicken lines revealed 2.8 million SNPs and a SNP rate of approximately ~5 SNPs per kb. Suprisingly, the SNP rate is of a similar magnitude between domestic and red junglefowl as between different domestic breeds (silkie, broiler and layer).

The chicken was the first domestic animal to be sequenced, but it has been shortly followed by the dog and cattle genomes. Being the first sequenced avian species, the chicken sequence provide interesting information regarding conserved genes and regions, it may function as an evolutionary outgroup in mammalian studies and shed new light on vertebrate evolution.

History of the chicken linkage map

The first classical linkage map for chicken was published in 1930 by Serebrovsky and Petrov and it contained 16 markers on eight linkage groups. The second map was published shortly thereafter by Hutt (1936). These early maps were based on phenotypic markers in intercrosses between various chicken lines. Some of the first such phenotypic marker identified in chicken was the *Dominant white* colour loci (Bateson, 1902) and the sex-linked *Barred* locus on the Z chromosome (Spillman, 1909). A review of advances in chicken gene mapping was published by Romanov, Sazanov & Smirnov (2004).

The first linkage map with DNA markers consisted of 100 RFLP markers and was published in 1992 (Bumstead & Palyga). Since then three major mapping populations have been used for linkage mapping in chicken; the East Lansing population (Michigan State University, USA), the Compton population (Compton Laboratory, United Kingdom) and the Wageningen mapping population (Wageningen, Netherlands). The linkage maps were integrated to form a consensus map by Schmid *et al.* (2000). Further development of the chicken linkage map is needed both in order to improve the resolution of the chicken genome sequence assembly and to identify genetic markers on microchromosomes.

Previous QTL experiments

Several intercrosses have previously been used for QTL mapping in chicken. For example, a broiler and layer intercross was generated to map QTL for growth and egg production traits (Ikeobi *et al.,* 2002; Sewalem *et al.,* 2002). QTL analyses for egg-production traits was performed in an intercross between divergent layer lines (Sasaki *et al.*, 2004; Tuiskula-Haavisto *et al*., 2002, 2004). QTL analyses have been reported for an intercross between two White Leghorn lines, one resistent and one susceptible to Marek's disease (Yonash *et al*., 1999). QTL analysis results have previously been reported for growth (Tatsuda & Fujinaka, 2001; Ikeobi *et al*., 2002; Sewalem *et al*., 2002; Van Kaam *et al*., 1998, 1999a,b; Jennen *et al*., 2004; ;Wardecka *et al*., 2002), egg production (Sasaki *et al*., 2004; Tuiskula-Haavisto *et al*., 2002, 2004), immune traits (Yonash *et al*., 1999; Zhu *et al*., 2003, Siwek *et a*l., 2003a, b, 2004,) as well as behavioural traits (Buitenhuis *et al*., 2003a, b, 2004; Schütz *et al*., 2003; Keeling *et al*., 2004). Recently, efforts have been made to map pairs of interacting loci (epistasi) in chicken (Carlborg *et al.*, 2003; Carlborg *et al*., 2004). Furthermore, a study based on an intercross between two divergent egg-laying chicken lines recently revealed QTLs with parent-of-origin effects (Tuiskula-Haavisto *et al*., 2004). The results are intriguing and address the question whether imprinting is present in birds, a mechanism that has not been shown in any avian species so far. Further analyses are needed to investigate the phenomenon.

Aims of the thesis

The objectives of the study have been to:

- Construct linkage maps for Quantitative Trait Loci (QTL) analyses
    - Assign previously unassigned markers to the chicken linkage map
    - Estimate recombination frequencies on micro- and macrochromosomes respectively
- Dissect the genetics of growth and fatness in chicken
    - Map QTL affecting growth and egg production in an intercross between White Leghorn and the ancestor of domestic chicken breeds, the red junglefowl
    - Elucidate the genetic components of growth and growth-related traits in an intercross between two extreme growth lines of chicken by QTL analysis

Methods

The identification of genes influencing complex traits is a challenge in any species. Therefore, it is common to apply a more general approach to search for genomic regions harbouring genes affecting a trait, rather than predicting genes based on previous knowledge of the trait and then set out to prove involvement of a candidate gene. The method identifies Quantitative Trait Loci (QTL), e.g. loci affecting a quantitative trait. With this approach it is possible to identify unexpected genes involved in the regulation of a trait, since no prior knowledge about the gene function is needed. Figure 3 provides an overview of the different steps in a typical QTL study using an experimental intercross.

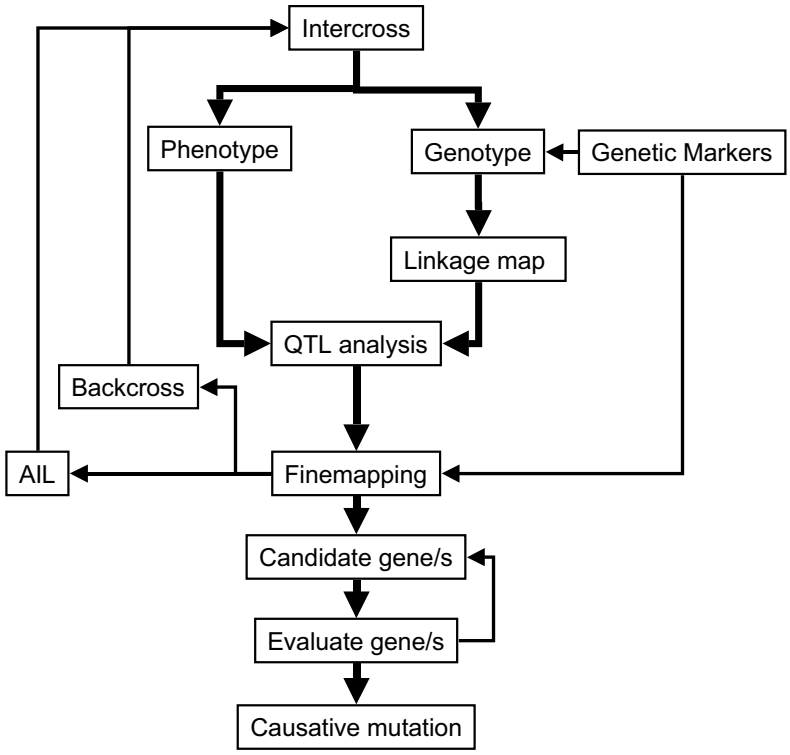

Figure 3. Flowchart showing a typical positional cloning experiment using an F_2 intercross. Thick arrows represent the shortest approach, but many of the alternative routes (thin arrows) are usually needed to identify the causative mutation. AIL stands for Advanced Intercross Line (Darvasi & Soller, 1995).

Pedigrees

Pedigree information is a prerequisite for QTL mapping. The type of pedigree varies depending on the purpose and organism used in the study. In humans, existing family material is used and in the specific breeding scheme used in cattle (few sires with many offspring) facilitates the use of the granddaughter design for QTL mapping. In smaller animals, specific intercrosses can be generated to maximize the power to detect QTL. This is costly and time-consuming in larger animals with longer generation times. One useful method to generate powerful mapping pedigrees is to establish an F_2 intercross by crossing genetically divergent breeds (Figure 4). Phenotypes are scored in the F_2 generation of the pedigree to allow mapping of trait loci.

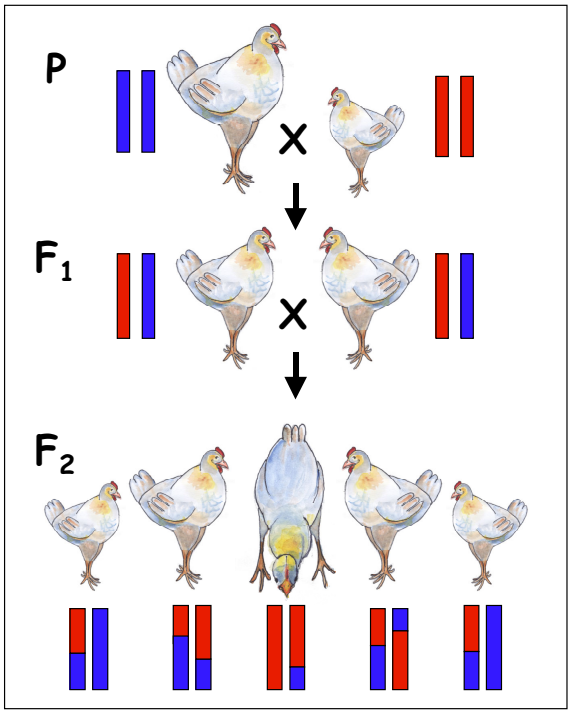

Figure 4. Overview of an F_2 intercross between two divergent chicken breeds. The bars represent chromosome pairs and the colours show from which parental line a chromosomal region stem. (Chicken illustrations: Brita Jacobsson)

Genetic markers

Genetic characters that are easy to score and show a simple inheritance are useful as genetic markers. A genetic marker may be phenotypic traits such as coat colour or comb size, single nucleotide polymorphisms (SNPs), restriction fragment polymorphisms (RFLPs) or simple repeats in form of mini and microsatellites. Today, the most commonly used genetic markers are microsatellites and SNPs. Microsatellites are long stretches of short repeats. They are often highly variable in length within populations, they occur fairly evenly spread throughout genomes and may have many alleles. SNPs are bi-allelelic polymorphisms that are becoming increasingly important due to powerful SNP discovery projects. Multiallelic microsatellites are more informative than bi-allelic SNPs and therefore, several SNP markers need to be genotyped to reach the information of one microsatellite. To be able to trace the inheritance of marker alleles in a pedigree, the markers must be informative. In an experimental intercross, this means that the founder lines must show high fixation for different alleles at the marker.

Linkage analysis

Transmission of genes from parent to offspring occurs through meiosis where chromosomes are duplicated and the pairs are separated to form gametes. Recombination, or crossingover, between the chromosome pairs occurs during meiosis (Figure 5). This results in new combinations of alleles on the chromosome. The recombination frequency between two loci is a function of the distance between them. The closer two loci are on a chromosome, the less likely it is that a recombination event will take place between them. Therefore, it is possible to estimate the distance between two markers by measuring the recombination fraction between them (Figure 6). Markers on different chromosomes, or far apart on the same chromosome, have a recombination frequency of 0.5. If one could assume that only one recombination event occurs between two loci, the recombination fraction would be a direct measurement of genetic distance. But this is not the case and several recombination events may occur between two loci on the same chromosome. Map functions have been developed to compensate for such double recombinants. The most commonly used map functions are the Haldane (Haldane, 1922) and Kosambi (Kosambi, 1944) functions.

A linkage map consists of marker loci in order on a chromosome and the map distance between the markers. The distances are given in centiMorgan (cM, one cM is equal to one recombination event in 100 meioses) and are calculated using one of the map functions. Linkage maps are constructed by linkage analysis in pedigrees where

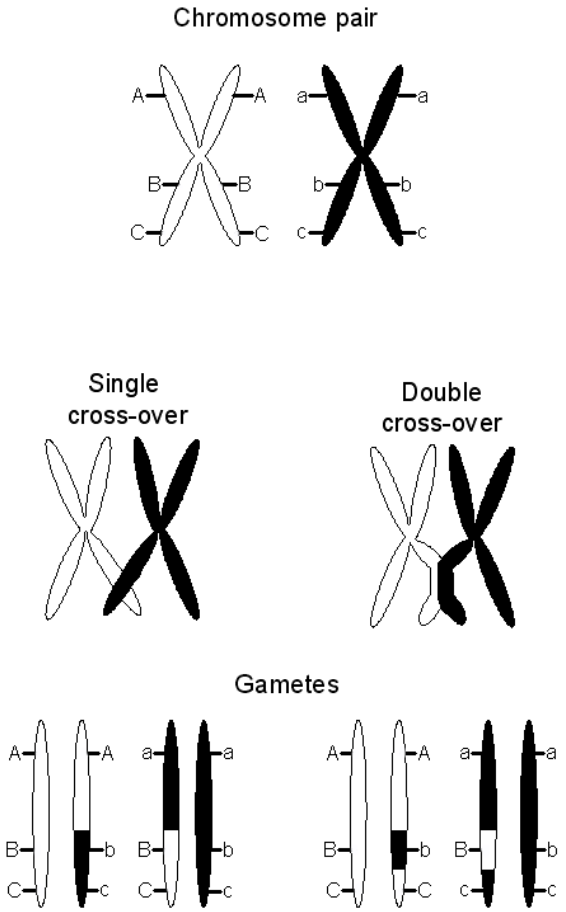

Figure 5. Illustration of a single (left) and a double (right) recombination event.

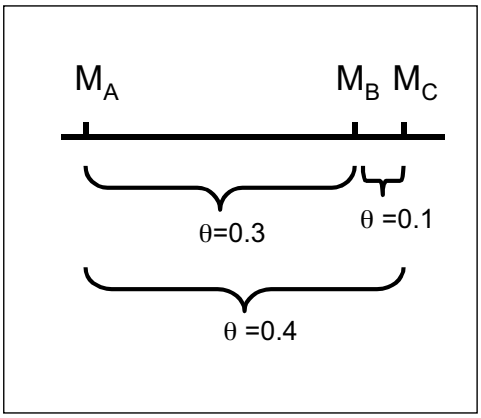

Figure 6. Recombination fraction is an indication of the genetic distance between marker loci. Here, three markers (A, B and C) have been genotyped and the recombination fraction (□) has been estimated. Observe that a higher recombination fraction corresponds to a longer distance.

a number of markers have been genotyped. In large pedigrees consisting of many individuals and where many markers have been genotyped, computer programs are used to construct linkage maps. The CRIMAP software (Green, Falls & Crook, 1990) is often used for linkage analysis in intercrosses between outbred populations.

Data handling

In large QTL experiments, many F_2 individuals are generated, phenotyped and genotyped for many markers. This generates large amounts of data. To keep track of our data we have used a database (Arexisdb). Apart from storing data, the Arexisdb is searchable, can display data in useful ways and perform inheritance tests for genotype data. Data can also be exported for use in other programs, for example the CRIMAP software (Green, Falls & Crook, 1990).

QTL analysis

Traits such as length and growth show a continuous rather than a discrete variation. These quantitative traits are controlled by many loci. A Quantitative Trait Locus (QTL) is defined as a chromosomal region harbouring one or several genes that influence a quantitative trait. Analyses to identify QTL are based on co-segregation of markers and genes affecting phenotypic trait variation.

In a classic single marker QTL analysis, the association between marker genotypes are compared with phenotypes in a single-locus test. In order to exploit the full potential of QTL analyses, Lander & Botstein (1989) proposed a QTL mapping method where the linkage map was utilized to estimate QTL effects also between markers in crosses of inbred lines. Haley, Knott & Elsen (1994) extended the interval mapping method in order to apply it to intercrosses between outbred populations. The method allows for segregation at marker loci but assumes that the QTL is fixed for different alleles in the founder lines.

Through the marker analysis it is possible to trace recombination events and determine the founder origin for each F_2 individual at every position across the genome. The probabilities of being homozygous for either founder allele or heterozygous are estimated for each individual at every cM. The measured phenotypes are regressed onto the estimated genotype probabilities and a statistical test is performed to test how much of the phenotypic variation is explained by segregation at each position. Basically, the phenotype variation is compared to the inheritance pattern at marker loci. Matching inheritance patterns gives a signal of a QTL.

Genetic models are built to estimate additive and dominance effects. A genetic model describes the phenotype of each individual as a function of the mean of all individuals, fixed effects, covariates and residual variance. Fixed effects are included to compensate for phenotypic differences due to a fixed factor. For example, body weight often differs significantly between the sexes. Other examples of fixed effects are family or feeding program. Furthermore, certain traits tend to co-variate with each other. Typically, body fat varies depending on an individuals' body weight. To analyse body fat, body weight is included as a covariate and each body fat score is corrected for body weight of the same individual.

In interval mapping, a large number of statistical tests are performed (one test per trait and cM) and thus the risk of detecting false QTLs increase. Therefore, the significance threshold levels need to be adjusted for multiple testing. This is often done by repeatedly randomizing the data (disconnect the relationship between genotype and phenotype) as suggested by Churchill & Doerge (1994). Thresholds are normally set to 1% or 5% genome wide significance, where 1% or 5% of the detected QTLs are expected to be false positives. Significance levels for suggestive QTL are often set to 20% genome wide or 5% chromosome wise thresholds.

Fine mapping

Fine mapping of QTLs can be performed by increasing the marker density in QTL regions. However, a satiety threshold is reached when the marker density is so high that no recombinations are detected between marker loci and the QTL. When the fine mapping is failing to reduce the region one of two approaches are often used to reduce the region further. First, an Advanced Intercross Line (AIL, Darvasi & Soller, 1995) may be maintained. In an AIL, the F_2 generation is further intercrossed to produce subsequent generations, and recombination events are collected over several generations. These recombination events can be utilized to fine map QTL regions provided that there are sufficient markers available in the region to distinguish between the different haplotypes. Secondly, a backcross is generated by crossing the F_2 generation individuals back to one of the founder lines. The F_2 individuals are carefully chosen to represent different genotypes in the QTL region and backcrossed to determine whether they carry the QTL allele or not. With this information it may be possible to exclude regions that do not harbour the QTL. In a backcross experiment, recombination events are accumulated for one locus at a time, whereas in an AIL recombination events are accumulated for all loci.

Positional candidate genes and causative mutations

The regions identified in a QTL analysis are often large and fine mapping of the region by generating a dense linkage map is often needed. A search for candidate genes within the QTL region may result in one or several candidates, based on previous functional studies. Evaluation of candidate genes is a laborious task and many molecular methods are often used before a certain gene is searched for causative mutations (QTN, Quantitative Trait Nucleotide).

Results and discussion

We have generated two intercrosses between divergent chicken lines in order to dissect genetic components of growth related traits. The two intercrosses allowed for comparisons between different intercross strategies, marker information content and base population effects on the results.

The first intercross is between the red junglefowl and White Leghorn. Domestic chickens show an enormous phenotypic diversity with regards to size, production traits, plumage colour, behaviour and other traits and they provide a unique opportunity to study genetic variation (Andersson, 2001). The red junglefowl (Fig 1A) is the ancestor of all domestic chickens and it is clear that they must comprise an enormous genetic variability to be able to respond so dramatically to selection. Although new mutations will contribute to the selection response, it is clear that much of the variation was present in the ancient red junglefowl.

We were interested in studying the differences between the red junglefowl and a domestic chicken line and we choose the commonly used egg producing White Leghorn breed (Fig 1) to represent the domestic chicken. The egg-layer is an interesting breed as they have radically different reproduction patterns (continuous instead of seasonal egg production) and the broodiness (desire to hatch eggs) is minimized compared to the red junglefowl. Moreover, a high egg production demands an effective calcium metabolism for production of egg shell. Layer chickens may therefore provide a model system for osteoporosis. Our intercross experiment aimed to elucidate the genetic changes that have taken place during domestication and in particular regarding the genetics of growth and egg production traits. One red junglefowl male and three White Leghorn females were crossed to generate four F_1 males and 37 F_1 females. By intercrossing the F_1s, 851 F_2 individuals were obtained.

The second intercross is between a high and a low body weight selection line (described in the Introduction). The lines are interesting models for growth and appetite traits as they differ almost nine-fold in weight at 56 days of age. They also show a dramatic difference in appetite. The selection lines, and the intercross between them, provide a unique opportunity to understand the genetic response to strong selection on one trait only (body weight at 56 days). Moreover, the lines present an animal model for metabolic disorders in humans. The mapping population was based on a reciprocal intercross between the 41st generation of the high and low selection lines. In total, 59 parentals were intercrossed so that 10 high line males were mated to 22 low line females and 8 low line males to 19

high line females. From the intercross 8 F_1 males and 75 F_1 females were used to produce 874 F_2 individuals.

I. Marker analysis and construction of linkage maps (Paper I and II)

For the red junglefowl (RJF) x White Leghorn (WL) intercross, 105 markers (100 microsatellites, 4 SNPs and 1 phenotypic trait) were genotyped in the pedigree and they formed 27 linkage groups including the Z chromosome. Twenty marker gaps were larger than 40 cM. Average information content at marker positions was 0.77 and the average marker distance was 24.3 cM. A test for differences in map length between the sexes showed some chromosomes where the female map was longer and some where the male map was longer. No clear overall trend was found.

A linkage map comprising linkage groups for 25 autosomes and the Z chromosome was constructed for the intercross between the high (H) and low (L) weight selection lines. A total of 145 genetic markers were used, of which 14 had not been mapped to a chromosomal location before. The total map length was 2521.9 cM with 17 gaps greater than 40 cM. The average information content of 0.55 was increased to 0.72 when information from adjacent markers was included.

Linkage map evaluation

The constructed linkage maps both cover ~80% of the genome (80.7% for H x L and 80.6% in RJF x WL), given that each marker covers 20 cM in each direction and the total chicken linkage map is 4000 cM. To obtain these numbers, the gap lengths exceeding 40 cM were subtracted from the total map lengths and an additional 40 cM was added per chromosome to account for the 20 cM that each end-marker covers. These values were divided by 4000 cM to estimate the marker map coverage.

A way of evaluating the quality of a linkage map is to compare the genetic distances with other published maps. In general, few genotype errors are expected if the map length is of the same length or shorter than other published maps since genotyping errors inflates estimated map distances. When comparing the genetic map length with the consensus map in Schmid *et al*. (2000), no overall difference was obtained for the H x L intercross (the H x L/consensus ratio equals 1.00). However, chromosome 3 is 40% longer in our map and chromosome 7 is 40% shorter than the consensus map. Comparisons could only be performed for markers on 7 linkage groups and the Z chromosome because many of the genotyped markers in this intercross are not present or are assigned to a large region on the Consensus map. The comparison shows that the genotype data are

overall reliable. In the RJF x WL intercross 15 linkage groups and the Z chromosome could be evaluated since the markers used in this intercross were generally chosen among those with a known position in the consensus map. A 0.93 ratio between RJF x WL to the consensus map lengths indicates this linkage map is of good quality. The sex chromosome is 30% shorter in the RJF x WL population compared to the Consensus map.

Assignment of markers

With the chicken genome sequence available, assignment of markers to chromosomal locations may seem less important. Nonetheless, the genome sequence is far from complete with a relatively large proportion of the sequence information unassigned to chromosomal locations. Thus, linkage mapping and assignment of markers to the genome is needed to improve the sequence assembly. Here, we have assigned 14 previously unmapped markers to chromosomal locations and the information was used in the assembly of the chicken genome (Hillier *et al*., 2004).

Information content

It is important that the information content (IC) of markers is high both for construction of linkage maps and for further QTL analyses. In the perfect situation all markers should be fixed for different alleles in the parental lines. In order to choose highly informative markers, many microsatellites were initially tested, from which a subset was genotyped in respective pedigree. In the RJF x WL intercross all founders were used for marker tests and in the H x L intercross five individuals from each parental line (17%) was used to test information content. When comparing the number of informative and uninformative microsatellite markers in the two intercrosses, there is a clear difference. Only 8.9% of the markers were fully informative in the high and low lines compared to 36.5% in the red junglefowl and White Leghorn. This is reflected in the average information content among the markers used for linkage analysis (0.55 and 0.77 in H x L and RJF x WL respectively) where we observed a four-fold higher ratio of fully informative markers in the RJF x WL compared to the H x L intercross. The differences can be explained in the context of the strikingly different genetic backgrounds of the founder populations.

Red junglefowl and White Leghorn stem from the same population prior to domestication. It is likely that the populations have large allele frequency differences at many loci as a result both of selection/domestication and by random fixation which has accumulated over many generations since the common ancestor. Moreover, the intercross is based on only four individuals, which reduces the amount of variation within each population to that

present in the four randomly selected individuals. The single red junglefowl male used in the pedigree has a maximum of two alleles at each loci and four alleles was the maximum number of different alleles observed among the three White Leghorn females.

The high and low selection lines both originate from the same base population 41 generations prior to the intercross and they are therefore expected to share alleles at loci that has not been under selection. A smaller number of alleles are expected to be fixed by chance due to the relatively few generations that have passed since the start of the selection experiment. Furthermore, approximately 30 individuals from each line were used to generate the mapping population. The larger number of parentals used in this intercross will reflect the population allele frequencies more accurately, but heterogeneity within the populations makes it more difficult to find highly informative markers.

Recombination rates on macro- and microchromosomes

In the H x L intercross, genetic and physical distances were compared in order to estimate the recombination rate in macro and microchromosomes respectively. The comparison revealed a 3-fold higher recombination rate on microchromosomes (~105 kb/cM) compared to macrochromosomes (~340 kb/cM). These numbers can be compared to the estimations performed in the chicken genome sequence paper (Hillier *et al*., 2004) where 1 cM equals ~ 156.3 kb and 357.1 kb on micro and macrochromosomes respectively (median values). Our estimation for microchromosomes is considerably lower than that in the sequence paper.

II. QTL mapping of complex traits

A number of phenotypic traits were recorded in the two intercrosses. For the RJF x WL intercross, growth, egg-production, bone traits as well as behavioural traits and plumage colour were scored. Here, we report results for growth and egg production traits.

In the H x L intercross, body weight at hatch, 14, 28, 42, 56 and 70 days of age were scored, as well as metabolites in blood (insulin, glucagons, IGF1, glucose, cholesterol and triglycerides), body composition traits (abdominal fat, breast muscle, lung, spleen, bursa and shank), immune response to sheep red blood cells (SRBC), packed cell volume (PCV) and blood protein.

QTL analyses in the red junglefowl x White Leghorn intercross (Paper II)

The approach of intercrossing a domestic species to its wild ancestor has been applied before when the Large White pig breed was crossed

with Wild Boar (Andersson *et al*., 1994; Knott *et al*., 1998). The intercross was initiated in 1989 and it is still generating interesting data. A regulatory mutation in intron 3 of IGF2 affecting pig muscularity was recently published based on this intercross (Van Laere *et al*., 2003). However, our study is the first time such an intercross has been performed in chicken to map QTL.

Almost 70% of the growth difference between the parental lines is explained by only four major loci (*Growth1*, *Growth2*, *Growth8*, *Growth13*). This is a surprising finding and stands in sharp contrast to the infinitesimal model in quantitative genetics, where an infinite number of loci each contributing with a small effect, describe the quantitative trait (Lynch & Walsh, 1998). The QTLs explain more of the variation in late growth compared to early growth which may imply that a smaller number of loci are involved in late growth. Furthermore, a genome wide search for epistatic QTLs in the RJF x WL intercross revealed more epistatic interactions between loci in early than late growth (Carlborg *et al*., 2003). The largest QTL, *Growth1*, solely explain 20% of the variation between the parental lines. Only one other study has identified a QTL in this region (Sewalem *et al.*, 2002) which is surprising considering the large effect on growth. An explanation for this may be that it reflects a QTL that was fixed early during domestication and therefore is fixed in all domestic lines used in other QTL studies.

Comparison of QTLs between studies are difficult to perform due to poor precision in QTL mapping and QTLs with large confidence intervals in initial genome scans. The marker density is too sparse for accurate comparisons. However, some overlap with previous studies was noted. Interestingly, *Growth2* overlaps with QTLs for carcass percentage (Van Kaam *et al.*, 1999), 9 week body weight (Sewalem *et al*., 2002), body fat (Ikeobi *et al*., 2002), abdominal fat (Jennen *et al*., 2004) and body weight (Zhu *et al*., 2002), although the fat QTL identified by Ikeobi *et al*. is only significant at the suggestive level. If these QTLs represent the same loci, it would be highly interesting, as it affects many aspects of body size. Nevertheless, further fine mapping of the regions is needed before any firm conclusions can be drawn. Also the possibility to search for candidate genes within a QTL region is greatly affected by the confidence interval of the QTL. Large QTL regions increases the number of possible candidate genes and fine mapping of the regions is necessary unless the causative mutation occurs in an obvious and well-studied candidate gene within the confidence interval.

This study shows, it is possible to identify highly significant QTL when the study is sufficiently large. In this case, an F_2 population consisting of ~800 individuals were sufficient to identify QTLs explaining a large proportion of the growth difference between the parental lines. For average egg weight we identified three highly

significant QTLs, one of which is co-localised to the major growth QTL on chromosome 1. An additional, suggestive QTL, is localised on chromosome 3. Egg-production traits may be strongly correlated to bone traits, as the calcium metabolism necessary for egg shell takes place in bone. *Growth1* is highly significant for growth traits. However, other traits give significant signals in close vicinity of this QTL. These traits include egg-production traits, tonic immobility (Schütz *et al*., 2004) and bone traits (unpublished data). One can hypothesize that this major QTL has a pleiotropic effect on many traits. However, further studies are needed to reveal the genetics behind this QTL.

Several studies have been published based on this intercross. Carlborg *et al*. (2003) studied epistatic interactions between pairs of loci and a significant QTL for tonic immobility was identified by Schütz *et al*. (2004). Tonic immobility is considered a fear response in chicken, and is characterized by the chicken acting dead when turned on its back. The White Leghorn stay in this immobilized state for a longer period of time than the red junglefowl. Plumage colour is another successfully studied trait, as several classical plumage colour genes segregate in the pedigree. The *Extension* locus was studied by Kerje *et al*. (2003) and the gene responsible for *Dominant White* colour was identified in the intercross (Kerje *et al*., 2004). Furthermore, the *Barred* and *Silver* colour loci are currently under investigation. Fine mapping of the *Growth1* QTL is underway and four backcrosses have been generated to position this major QTL with great confidence.

QTL anasyses in an intercross between High x Low weight selection lines (Paper III and IV)

Intercrosses between chicken lines divergently selected for immune traits have previously been reported (Yonash et al., 2001; Siwek *et al.*, 2004). However, this is the first experiment where two selection lines divergently selected for body weight have been used for QTL mapping in chicken. Similar QTL studies have been conducted in mice selected for high and low fat content (Horvat *et al.*, 2000) and in mice bi-directionally selected for activity (Henderson *et al*., 2004) as well as in *Drosophila* selected for high and low bristle numbers (Gurganus *et al*., 2000).

We report the results from genome scans to reveal growth and growth related QTLs in two papers. QTLs for growth as well as anorexia, packed cell volume (PCV), blood protein and immune response following immunisation with sheep red blood cell (SRBC) are discussed in paper III, whereas body composition (abdominal fat, breast muscle, shank, lung, bursa and spleen) and metabolic traits (blood glucose, cholesterol, triglycerides, insulin and IGF1) are reported in paper IV.

Growth QTLs explain a small part of the phenotypic difference

The 13 growth QTLs (*Growth1* to *Growth13* in Table 2, Paper III) identified in this study explain approximately 50% of the difference between the parentals at 56 days of age (selection age) and around 13% of the residual variance in the F_2 population. Nevertheless, these numbers are approximate, since all QTLs were not significant at 56 day body weight, *i.e*. they were based on estimations of QTL effects that may have been inflated for a number of reasons (see Discussion, paper III). All 11 QTLs that show additive effects were analysed in a joint least square analysis for body weight at 56 days. Five of the QTLs were significant at the 5% genome wide level for at least one trait. It is interesting to estimate the effect on 56 day body weight of the identified QTLs, as we expect all QTLs should have an effect on body weight at selection age (56 days), or they would not have been affected by selection. The total amount of variation explained by the QTLs is considerably lower compared to the red junglefowl intercross where almost 70% of the variation was explained by four major QTLs. The genome scan is, however, not complete as we are lacking markers on 13 microchromosomes and the marker coverage is sparse in some regions. These uncovered regions may contain QTL with large effects. A genome wide search for epistatic pairs of QTLs is underway and Carlborg *et al.* (unpublished data) has found that epistatic interaction among some of the QTLs detected in this study play an important role in this pedigree. Further exploration of models and sex-specific analyses (discussed below) may also contribute significantly to the amount of variance present in the F_2.

Metabolic and body composition traits

QTL analyses for body composition traits revealed four highly significant QTLs, two for breast muscle and two for shank weight. Two of the QTLs are colocalised with *Growth1* QTL on chromosome 1. The *Growth1* QTL shows at least suggestive significance for several traits, including growth from 56 to 70 days of age, abdominal fat together with breast muscle and shank weight. Interestingly, a QTL for abdominal fat at nine weeks of age was recently identified in a broiler x broiler cross in the vicinity of *Growth1* (Jennen *et al*., 2004). The QTLs for shank weight and muscle mass explains a much larger proportion (up to 13% for the shank QTL on chromosome 1) of the residual variance than what we have seen for growth. The finding is fascinating and may reflect that the genetics behind these traits are less complicated and regulated by a smaller number of genes than growth. No QTL was identified for anorexia, packed cell volume (PCV) or immune response to sheep red blood cells. In this study, metabolic traits include plasma concentrations for insulin, glucagons, IGF1, glucose, cholesterol and triglycerides. One QTL for glucose reached 5% genome-wide

significance and other QTL for metabolic traits only reached suggestive significance. Many of the metabolic QTLs are co-localised with growth QTLs, which adds reliability to the identified loci. The genetic architecture regulating these traits may be complex and future studies using more complex genetic models would be interesting.

Sex specific QTL

In order to understand the nature of identified QTLs, we performed sex-specific analyses at QTL positions. For a number of traits, the F-ratio went up considerably when the analysis was performed on one sex only. For example, the suggestive QTL for shank weight on chromosome 26 (F-ratio 6.7) reaches 5% genome wide significance (F-ratio 9.6) when the analysis is performed on females only, despite only half as many data points are included in the analysis. Also, a QTL for cholesterol on chromosome 3 reaches 5% genome wide significance (F-ratio 8.8) in females only. Further evaluations of the significance of the interaction with sex are needed.

Anorexia

Anorexia has been observed in the low weight selection line since the 25^{th} and 26^{th} generation. A percentage of the chickens die early post hatch because they never seem to start feeding or feed inadequately for survival. In generation 41, which was used for the F_2 mapping experiment, approximately 26% died early. No bird in the F_1 generation died, but 176 out of 974 birds (18%) died before 10 weeks of age in the F_2 generation. Approximately 80% of these early deaths occurred within the first 5 weeks (Table 3). In subsequent intercross generations 4.1% died in the F_3, followed by 3.4%, 14.4%, 7.8% and 3.6% in generations F_4 through F_7. The percent fluctuations reflect those seen in the low line (Lacy *et al.*, 1987), although these are not as dramatic in the intercross.

The incidence of anorexia in the low weight selection line and in the F_2 generation of the intercross is intriguing and the fact that we do not find any QTL for the trait may, as discussed in paper III, depend on any of several reasons. Table 3 shows the mortality distribution among the F_2 individuals per week. The time when most individuals die is in week 4 and 5 and these individuals must clearly be feeding at least to some degree, as the yolk sac will only last for about a week. This supports our hypothesis of a threshold effect where the appetite shows a continuous distribution rather than an on/off mode. It may also imply that the chickens die from different reasons at different ages. The chickens that die soon after hatch may do so due to some genetic factor different from those that dies later. The chickens that fail to reach sexual maturity can be brought into egg-production by force-feeding (Zelenka *et al.*, 1988), which clearly indicates impaired appetite control consistent with the finding that

electrolytic lesion of the hypothalamic regions causes the low line to increase feed intake and gain weight (Burkhart *et al*., 1983).

Table 3. Number of birds that died early (by week) and the number of birds which we have DNA samples from.

Week	No of deaths	Sampled
1	26	0
2	9	0
3	16	1
4	44	1
5	47	26
6	14	14
7	8	7
8	5	5
9	3	2
10	4	4
Total	176	60

We performed segregation distortion (SD) analyses in order to detect underrepresented alleles at certain loci. This would point towards incidence of anorexia as marked by certain lethal alleles being absent among the sampled individuals. However, the analysis did not result in more SD signals than expected by chance. Furthermore, SD was significant in only one QTL region on chromosome 5, although a small trend for higher survival in individuals carrying the high weight line alleles in QTL regions were noted.

Allele frequency and localisation of QTL

As mentioned before, it is relatively difficult to identify informative markers in this intercross, since the founders were part of the same population 41 generations prior to the intercross. We hypothesise that loci which has responded to selection are likely to be fixed for different alleles at nearby microsatellite markers. In fact, it may be possible to identify selection-responding loci by simply screening the founder populations for allele frequency differences across the genome. We tested this by investigating whether markers in the region flanking a QTL show high allele frequency differences due to hitch-hiking. Fst is an estimate of genetic divergence commonly used by population geneticists (Weir & Cockerham, 1984). We observed a clear tendency for correlation between high Fst values between the founder populations and the location of QTLs. Among the 20 markers that has an Fst value > 0.8, five markers were less than 10 cM from a QTL peak. In comparison, seven out of 130 markers with an Fst < 0.8 were positioned less than 10 cM from a QTL peak. This pilot study shows that it may be highly rewarding to study allele frequency changes along chromosomes between the high and low weight selection lines in much more detail.

III. Evaluation of a candidate gene (Paper V)

Chromosome 20 harbours a QTL (*Growth12*) affecting growth from 0-14 days and body weight at 14 days of age. The gene coding for *melanocortin receptor 3* (*MC3R*) is present within the confidence interval of the QTL. *MC3R* has previously been shown to affect body weight in mammals (Chen *et al*., 2000; Cummings & Schwarz, 2000; Dubern *et al*., 2001) and it is thus an obvious candidate gene for the QTL.

Following our pilot study with Fst-values, we expect the lines to be fixed for different alleles at the QTL and that this fixation gradually disappears with the distance from the QTL due to recombination events and decreasing selection pressure. Therefore, by studying the allele frequencies across the QTL region, it may be possible to estimate the position of the QTL further. Here, we have used the Fst estimates for markers across the QTL region to quantify the proportion of genetic variation that lies between the two selection lines (Figure 2, Paper V). An Fst value equal to 1 shows that the marker is completely fixed for different alleles and that all variation lies between the populations. Similarly, an Fst of 0.3 indicates that 30% of the variation is between lines and 70% of the total variation is present within lines. We observe that the lines are completely fixed for different alleles at *MC3R*, but not in the flanking markers located 400 kb (*HGEN003*) and 100 kb (*HGEN002*) on either side of the gene. In the low line, the flanking markers are fixed for one allele, but this allele is also present among the high line animals, indicating a stronger selection pressure on this region in the low line compared to the high line. The finding supports the candidate gene status of *MC3R*.

Fine mapping of the region and sex-specific QTL analyses revealed a male-specific expression pattern for the QTL which has its peak close to the *MC3R* gene. Four SNPs were identified in the *MC3R* coding exon, but none of the SNPs change the amino acid sequence of the protein and they are thus unlikely to affect *MC3R* function. Expression studies in the founder lines in relation to *GAPDH* revealed a 1.8-1.9 fold higher expression in the low line. In order to examine this differential expression further we collected samples from F_1 individuals. F_1 animals are expected to be heterozygous for the *MC3R* allele which makes it possible to study relative expression of the two alleles within an individual. If the two alleles are transcribed in unequal amounts one can assume a cis-acting factor of proportionally large effect. We found a small (~5%), but significant cis-acting effect, which strongly suggests that the major part of the differential expression in the founder lines is explained by one or several trans-acting factors.

Although, our experiments indicate *MC3R* as a strong candidate for the QTL for early male growth on chromosome 20, refinement of the QTL borders need to be performed to exclude other genes in the region. There are 8 genes within a ~1 Mbp region between the flanking markers *HGEN003* and *HGEN002*. Among those, the *cytosolic phosphoenolpyruvate carboxykinase 1* (*PCK1*), positioned only approximately 600 kb from *MC3R*, is considered a candidate gene for type II diabetes mellitus obesity (Beale *et al*., 2004; Cao *et al*., 2004). *PCK1* is positioned at 11.1 Mbp (*MC3R* at 11.7 Mbp) and thus close to *HGEN003*. *HGEN003* is fixed for one allele in the low line, but segregation is present among the high line chickens.

Further expression studies are needed to evaluate *MC3R* expression levels in smaller brain regions, preferably hypothalamus only, and to test for trans-acting effects for differential expression. Finemapping of the region in our advanced intercross line (AIL, Darvasi & Soller, 1995) will aid in excluding other genes in the region. Furthermore, the 5´ and 3´ untranslated regions of *MC3R* has not been sequenced in the lines and there may be functionally important elements there that affect translation of *MC3R*.

Conclusions

- By construction of linkage maps (covering ~80%) we have been able to assign 14 previously unassigned microsatellite markers to the chicken linkage map. These have been used in the assembly of the chicken genome.

- Linkage analysis revealed a three-fold higher recombination rate on macro- compared to micro-chromosomes.

- A large proportion of the two-fold difference in growth between the red junglefowl and White Leghorn were explained by the QTLs we identified. In fact, four major growth regulating loci are responsible for 80% of the difference in males.

- A smaller proportion of the phenotypic difference was explained by the QTLs identified in the intercross between low and high weight selection lines, although the founder lines differ almost nine-fold in weight.

- Metabolic and body composition QTLs are often co-localised with growth QTLs, indicating there may be QTL with pleiotropic effects on many traits.

- A full genome scan with complete marker coverage may reveal additional QTL with small or large effects on growth in both intercrosses.

- The gene encoding *melanocortin receptor 3* (*MC3R*) is positioned within a male-specific QTL for growth on chromosome 20. The locus is fixed for different alleles in the two lines, whereas nearby markers do not show fixation. Expression studies indicate a small cis-acting effect on *MC3R* expression.

Future prospects

Important research questions are likely to be answered using the two intercrosses in the future. Examples of subjects that may be addressed are the process of domestication as regards to the RJF x WL intercross, population changes during strong selection (H x L intercross), genetic dissection of growth, behavioural and other traits for use both in chicken production and in human disease.

Ongoing

There are two major issues for successful QTL mapping experiments. First, the selected founder animals or pedigrees must exhibit divergent phenotypes as a result of different genetic background. Ideally, the founders should be reared under the same environmental conditions prior to intercrossing to ensure the phenotype is caused by genetics and less dependent on environmental factors such as temperature or feeding regimen. This is less important for some clearly genetic traits such as plumage colour, but more important for traits that are likely to be greatly affected by the environment. Examples of environment-sensitive traits are immune traits where exposure to different antigens may result in different phenotypes. The intercross should be setup to maximize the power of a QTL study and a sufficient number of F_2 individuals scored for relevant traits.

The second major concern with QTL studies is to reach a high resolution in order to minimize QTL confidence intervals and facilitate identification of causative genes and mutations. High resolution mapping may be reached by constructing dense linkage maps with many genetic markers. However, there may not be enough recombination events between marker loci and the QTL in the F_2 population for close fine mapping. To overcome this problem we have maintained advanced intercross lines (AIL) for both intercrosses. The AIL is in the F_5 generation for RJF x WL and in generation F_8 in the H x L intercross. Recently, 400 individuals from the H x L F_8 generation were raised and phenotyped for growth as well as metabolic traits. The population will be used for high resolution mapping of QTL regions. Also, it is possible to further investigate the reciprocal effect on some traits reported in paper IV.

Another approach to break up linkage between QTL and closely linked marker loci is to backcross F_2 animals to either founder line. With this method the QTL region is reduced by performing progeny testing for QTL segregation in backcrossed individuals in order to exclude regions flanked by markers that are not co-segregating with the QTL. This approach is being applied to fine map *Growth1* on chromosome 1 in the RJF x WL intercross where backcrosses to the

White Leghorn are performed (U. Gunnarsson *et al*., Väisänen *et al*.., data not shown).

Today we cover approximately 80% of the genome with markers in both intercrosses. In order to perform a complete genome scan, we need a more dense marker map. The genome sequence, 2.8 million described SNPs and a relatively dense microsatellite marker map are extremely useful resources for this task. Nevertheless, many microchromosomes lack markers and have not been assembled in the genome sequence. Further mapping is thus of importance, especially for michrochromosomes and the Z chromosome, where the assembly is sparse.

The large number of described chicken SNPs (2.8 million) is useful for several purposes. In analogy with the observation that marker loci are often fixed for different alleles within QTL regions, large scale screening of SNP markers in the founder lines of the H x L intercross may provide a useful tool for fine mapping of QTL regions and for identification of markers in previously uncovered genomic regions.

A study of pair wise epistatic interactions between loci in the high and low weight selection lines has shown that interactions between loci affects body weight at 56 days of age (Carlborg *et al.*, in preparation). Similar searches for interacting loci would be interesting to conduct for other traits. Further exploration of genetic modelling of the traits in QTL analyses including sex specific analyses and random family effects will be beneficial.

Microarray experiments have been conducted for both intercrosses (in collaboration with Sojeong Ka and Finn Hallböök) and positioning differentially expressed genes in relation to QTL regions may result in candidate genes for the QTLs. In fact, for the high and low lines approximately 40 of the 180 most differentially expressed genes (for which it was possible to determine the genomic position) are within QTL regions and among those some interesting candidate genes are currently being confirmed using RT-PCR techniques.

If differential expression is involved in the anorexic effect causing chicks to die soon post hatch in the low weight line because they fail to commence feeding, we expect to see differential expression in newly hatched chickens, but not at eight weeks (since the animals exhibiting the differential expression may be dead). Furthermore, since the phenotype is equally common among males and females we expect to see the differential expression in both males and females. It turns out that only five genes fulfil these criteria and one of the genes are in a region showing significant segregation distortion on chromosome five. Further studies of the gene and its potential to cause segregation distortion in the pedigree are underway.

Further ahead

To study how selection response affects a population it would be interesting to sample individuals from the high and low weight selection lines in 2009 (generation 51), ten years after the founders of the F_2 intercross were sampled. A subset of SNPs could be selected to study what effect another ten years of intense selection for 56-day body weight has had on allele and haplotype frequencies along the genome.

With regards to the anorexia trait, several interesting questions can be addressed in the future. We are currently collecting liver samples from 40 anorexic and 40 control individuals among the low selection line of generation 47. These may be used for example to analyse haplotype frequencies in regions that show segregation distortion and serve as controls for candidate genes for anorexia.

Discussions regarding set-up of a mapping population comprising up to 2000 F_{10} birds has been initiated. A mapping population of this size will allow for identification of QTL explaining a smaller part of the phenotypic variance between the two parental lines and our estimates of the QTL effects would be much more precise. For the H x L weight intercross, this would make a significant difference as most of the QTLs for growth explain only a small part of the residual variance (maximum 3.1%).

QTL regions without obvious candidate genes are difficult to explore and given the great advance in microarray technology and availability of chicken clones, QTL specific microarrays could be one option to identify candidate genes. Furthermore, once a hypothesis has been proposed on the basis of a QTL or QTN affecting brain function, it is possible to functionally explore the thesis using standard methods to introduce potential substances into the brain.

Genotyping is becoming less expensive with the advent of cheap SNP typing methods, which facilitate high resolution QTL scans at lower costs. However, maintaining experimental intercrosses and animal husbandry is still expensive. Therefore, availability of biological material is likely to set the limitations on future genetic studies and thus efforts should be made to build up biobanks of biological material as well as pedigree information.

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I

BREEDING AND GENETICS

Assignment of Fourteen Microsatellite Markers to the Chicken Linkage Map

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ABSTRACT A large mapping population, with 874 F_2 individuals, was generated by reciprocally intercrossing 2 chicken lines. A genetic map of 2,426.6 cM comprising 25 linkage groups was established based on 145 microsatellite markers. Chromosome locations were assigned for 14 previously unmapped markers. The marker *ADL0132* was previously mapped to chromosome 9; however, here close linkage to the *MCW0091* marker on chromosome 4 was found. With this exception, the derived linkage map was in excellent agreement with the chicken consensus map. A comparison with the chicken genome assembly (http://genome.ucsc.edu; February 2004) suggested a few minor errors in the assembly. A PCR-RFLP test was used to genotype a single nucleotide polymorphism in the melanocortin receptor 3 (*MC3R*) gene in the intercross, and pyrosequencing was used to map the genes for Hemopoetic Cell Kinase (*HCK*) and Bone Morphogenic Protein 7 (*BMP7*). The *HCK* and *BMP7* genes on linkage group E32 showed significant linkage to *MC3R* on the distal end of linkage group E47W24, consequently joining the 2 linkage groups. A comparison between the linkage data in the current study and the physical location of markers as revealed in the chicken genome sequence assembly (February 2004) showed a 3-fold higher recombination rate on microchromosomes than on macrochromosomes.

(*Key words*: chicken, linkage map, microsatellite, recombination rate, single nucleotide polymorphism)

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INTRODUCTION

A comprehensive genetic map is a prerequisite for mapping QTL. The first linkage map for the chicken reported by Bumstead and Palyga (1992) consisted of about 100 RFLP markers. Schmid et al. (2000) reported the first consensus linkage map for the chicken genome. Chromosomal locations for 1,965 markers forming 50 linkage groups were reported. In the Ark Database (www.thearkdb.org) there are 2,483 loci for the chicken, of which 435 are unassigned genetic markers. Identification of their chromosomal locations will aid in the search for QTL and in assembly of the chicken genome sequence.

A 3-generation pedigree was generated by intercrossing 2 lines of White Rock chickens divergently selected for juvenile body weight. The parental chicken lines were selected solely on body weight at 8 wk of age for 41 generations, which resulted in a 9-fold difference in body weight at age of selection (Liu et al., 1994). This resource pedigree will be used for mapping QTL controlling growth, appetite, and fat deposition. A linkage map based on genotype information from 145 microsatellite markers in the intercross is reported here.

MATERIALS AND METHODS

Chickens

The high (HW) and low weight (LW) selection lines were developed and maintained at the Virginia Polytechnic Institute and State University in Blacksburg, Virginia (Liu et al., 1994; Dunnington and Siegel, 1996). The founder animals originated from generation 41 of these long-term selection lines. The intercross was done reciprocally so that 10 HW males were mated to 22 LW females, and 8 LW males were mated to 19 HW females. From the F_1 generation, 8 F_1 males and 75 F_1 females were intercrossed, and 874 F_2 animals from a single hatch were used for the linkage study.

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Abbreviation Key: dNTP = deoxynucleotide triphosphate; HW = line selected for high weight; LW = line selected for low weight; SNP = single nucleotide polymorphism.

DNA Isolation, Marker Selection, and Genotyping

Blood samples were collected from all F_2 individuals, their parents (F_1), and grandparents (F_0). Seven microliters of blood were used for DNA isolation using the DNeasy96 Tissue Kit for mouse tails[2] with some minor modifications.

A total of 647 previously described microsatellite markers (www.thearkdb.org) were initially tested on a limited number of individuals (10 from the HW line and 10 from the LW line) to select the most informative markers to be included in the current study. A set of 145 markers was selected for the linkage study. A list of all markers used in the present study is provided in Table 1, including location on the constructed linkage map and information content in this intercross. The information content for each marker was calculated using the Web-based QTL Express software (Seaton et al., 2002; http://qtl.cap.edu.ac.uk/). Primer details for all microsatellite markers can be found at www.thearkdb.org.

The PCR amplifications of microsatellite markers were performed with fluorescently labeled primers. A total volume of 5 μL was used for the PCR reactions containing 1× PCR Buffer II,[3] 2.5 m*M* $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (dNTP), 1 to 5 pmol of each primer, 0.25 U of AmpliTaq Gold DNA polymerase,[3] and 20 to 50 ng of genomic DNA. The PCR reaction was started with an incubation for 5 min at 95°C to activate the polymerase, followed by a touchdown PCR cycle starting with annealing for 30 s at 65°C and decreasing by 1°C per cycle to 52°C. Forty cycles of PCR were performed with annealing at 52°C, denaturation for 45 s at 95°C, and extension for 30 s at 72°C. The last cycle included an extension step for 5 min at 72°C. The PCR products were denatured for 2 min at 95°C before electrophoresis in 4% polyacrylamide gels using an ABI377 sequencer[3] or a MegaBACE capillary instrument.[4] The results were analyzed with the Genescan[3] and Genotyper software[3] or Genetic Profiler.[4]

Single Nucleotide Polymorphism Analysis

Three additional loci *melanocortin 3 receptor* (*MC3R*), *hemopoietic cell kinase* (*HCK*), and *bone morphogenetic protein 7* (BMP7) were mapped in the intercross. The primers 5′-ACT ATT TTC TAT GCC CTC CTT TAC C-3′ and 5′-TGA AGC TGC TGT GTA GCT AT-3′ were designed from chicken sequence of *MC3R* (GenBank AB017137) and amplified a 628-bp fragment of the gene. The PCR was performed in a final volume of 20 μL containing 30 ng of genomic DNA, 15 m*M* Tris-HCl, and 50 m*M* KCl (pH8.0, GeneAmp Gold buffer[3]), 2 m*M* $MgCl_2$, 200 (M dNTP, 10 pmol of primer, 4% DMSO, and 0.5 U of AmpliTaq Gold DNA polymerase.[3] The reaction occurred in a PTC-200 thermal cycler[5] for 5 min at 95°C followed by 35 cycles of 45 s at 94°C, 45 s at 58°C, and 1 min 30 s at 72°C. An extra extension cycle at 72°C for 5 min was added in the end. The PCR products were purified using the QIAquick PCR purification kit[2] and sequenced with BigDye Terminator Cycle Sequencing chemistry.[3]

Sequencing of the *MC3R* fragment revealed a single nucleotide polymorphism (SNP) affecting a *Dde*I restriction site. The genotypes were scored using a PCR-RFLP assay, in which the allele from the HW line was cleaved into 3 fragments (321, 193, and 114 bp) and the allele from the LW line was cleaved into 2 fragments (435 and 193 bp). In the restriction reaction, 15 μL of the PCR products (generated as described above) was digested overnight at 37°C with *Dde*I.[6] The restriction fragments were separated by electrophoresis in a 1.5% agarose gel (Nusieve GTGAgarose[7]) and then the genotypes were scored.

The primers HCK_E9_F (5′-ATA CAT CAT CAC CGA GTT CAT-3′) and HCK_E10_R (5′-GCA GAG AAG TCG ATC AGC TTT-3′) amplify a 282-bp fragment of the *HCK* gene. Similarly, the primers BMP7_INTRON_5_F (5′-GGG CCA GCA TGT CAG ATT T-3′) and BMP7_INTRON_5_R (5′-GCA ATG TTG TGC GGT GAA A-3′) amplify a 150-bp fragment of the *BMP7* gene. Both *BMP7* and *HCK* were amplified in 10-μL reactions containing 20 ng of genomic DNA, 1× PCR II buffer,[3] 2.0 m*M* $MgCl_2$, 0.2 m*M* dNTP, 0.5 U of AmpliTaq Gold DNA polymerase,[3] and 10 pmol of each primer. The thermal cycling was performed in a PTC-200 machine[5] and started with 5 min at 94°C, followed by 40 cycles each consisting of 30 s at 94°C, 30 s at 56°C, and 45 s at 72°C and an additional cycle at 72°C for 10 min.

The PCR products were purified using the QIAquick PCR purification kit[2] and sequenced with BigDye Terminator Cycle Sequencing chemistry.[3] Sequence comparisons revealed SNP in both *BMP7* (nucleotide 96) and *HCK* (nucleotide 56).

Genotypes were scored using the pyrosequencing[8] method. Prior to pyrosequencing with the SNP Reagent Kit protocol,[8] the reverse primers were biotinylated to allow capture of single stranded products onto avidin-coated solid support. The pyrosequencing primers BMP7_E5_PYRO_F (5′-CAG ACA TAG GAA TTG GTA GA-3′) and HCK_E9_PYRO_F (5′- GCA CGG TGT GGG AC-3′) were designed with their 3′ ends just upstream of the polymorphic site. Ten picomoles sequencing primer was used in the pyrosequencing reaction. The result of the pyrosequencing assay was manually checked to enhance accuracy.

Statistical Analysis

Linkage maps for 25 autosomal linkage groups were generated using the CRI-MAP software (Green et al., 1990). The functions BUILD, FLIPS, FIXED, and CHROM-

[2]Qiagen, Valencia, CA.
[3]Applied Biosystems, Inc., Foster City,CA.
[4]Amersham Biosciences, Uppsala, Sweden.
[5]MJ Research, Inc., Waltham, MA.
[6]New England BioLabs, Inc., Beverly, MA.
[7]Cambrex BioScience Rockland, Inc., Rockland, ME.
[8]Pyrosequencing AB, Uppsala, Sweden.

TABLE 1. Genetic markers used to construct a chicken linkage map. Information content is given for each individual marker (IC^{mrk}) and for the marker position in the linkage map (IC^{pt})[1]

	Linkage map				Genome assembly	
Marker	Linkage group	Position (cM)	IC^{mrk}	IC^{pt}	Chromosome	Position (Mb)
MCW0168	1	0	0.3	0.6	1	1.0
MCW0248	1	3.2	0.4	0.6	1	0.6
LEI0209	1	29	0.3	0.5	1	16.6
MCW0254	1	56.5	0.9	0.9	1	26.5
UMA1.015	1	72.1	0.1	0.5	1	31.4
LEI0146	1	136.0	0.9	0.9	1	49.9
MCW0018	1	165.5	0.7	0.8	1	60.2
MCW0058	1	200.6	0.5	0.7	1	75.8
LEI0071	1	201.6	0.4	0.7	1 Random	1.4
ADL0367	1	250.2	0.9	0.9	1	82.3
MCW0327	1	256.9	0.2	0.7	1	85.4
MCW0268	1	264.9	0.4	0.7	1	87.4
LEI0108	1	266.4	0.2	0.6	1	87.7
MCW0200	1	290.6	0.2	0.4	1	101.5
ADL0353	1	328.9	0.6	0.8	1	114.1
ROS0310	1	328.9	0.6	0.8	1	114.1
MCW0036	1	344.6	0.9	0.9	1	118.3
LEI0169	1	357.3	0.4	0.7	1	123.6
LEI0107	1	390.4	0.8	0.9	1	132.4
LEI0079	1	414.2	0.8	0.9	1	151.0
LEI0162	1	430.2	0.3	0.7	1	157.0
ADL0245	1	438.4	0.6	0.7	1	160.0
LEI0134	1	523.6	0.8	0.8	1	179.2
GCT0001	1	529.0	0.3	0.7	1	184.4
ADL0190	2	0	0.2	0.3	2	14.7
MCW0247	2	11.3	0.2	0.3	2	18.8
ADL0176	2	69.9	0.5	0.7	2	36.2
MCW0063	2	72.8	0.6	0.8	2	37.1
MCW0062	2	113.8	0.8	0.8	2	54.9
MCW0293	2	123.5	0.9	0.9	2	58.2
MCW0130	2	127.5	0.9	0.9	2	59.4
LEI0096	2	167.9	0.7	0.8	2	69.5
LEI0248	2	169.9	0.3	0.9	2	71.7
ADL0157	2	178.6	0.8	0.9	2	78.5
MCW0179	2	187.2	0.5	0.8	2	84.2
MCW0087	2	189.0	0.5	0.8	2	84.8
UMA2080	2	208.3	0.7	0.8	2	93.6
LEI0147	2	216.4	1	0.9	2	97.3
MCW0234	2	248.1	0.9	0.9	2	110.4
GCT0002	2	269.6	0.1	0.4	2	116.8
MCW0245	2	293.4	0.9	0.9	2	126.5
LEI0070	2	306.1	0.9	0.9	2	132.7
MCW0320	2	318.3	0.9	0.9	2	143.7
LEI0031	2	325.9	0.6	0.8	2	136.3
MCW0311	2	326.8	0.7	0.8	2	135.9
MCW0169	3	0	0.6	0.6	3	10.4
MCW0222	3	72.3	0.3	0.4	3	19.4
ADL0155	3	114.2	0.3	0.5	3	32.6
ADL0371	3	136.2	0.5	0.6	3	39.5
MCW0004	3	154.6	0.5	0.6	3	48.6
MCW0224	3	211.7	0.9	0.9	3	75.4
ADL0024	3	221.3	0.6	0.8	3	79.0
MCW0207	3	236.5	0.6	0.7	3	88.3
LEI0065	3	262.1	0.4	0.5	3	99.1
ADL0143	4	0	0.4	0.7	4	3.6
ADL0317	4	11.5	0.7	0.8	4	3.3
ADL0145	4	88.7	0.6	0.9	4	17.5
MCW0251	4	90.3	0.9	0.9	4	20.3
ADL0144	4	122.8	0.8	0.9	4	36.6
MCW0091	4	128.1	0.5	0.8	4	38.9
ADL0132 *	4	130.0	0.3	0.8	4	39.8
LEI0125	4	136.4	0.8	0.8	4	43.8
LEI0122	4	139.2	0.2	0.8	4	41.2
LEI0076	4	182.5	0.5	0.7	4	60.9
LEI0148	4	206	0.9	0.9	UN†	
MCW0098	4	219.7	0.6	0.8	4	78.9
LEI0085	4	228.4	0.8	0.9	4	83.2
LEI0073	4	237.7	0.3	0.7	4 Random	1.1
LEI0116	5	0	0.4	0.4	NH‡	

Continued

TABLE 1 continued.

	Linkage map				Genome assembly	
Marker	Linkage group	Position (cM)	IC^{mrk}	IC^{pt}	Chromosome	Position (Mb)
MCW0193	5	56.0	0.6	0.7	5	12.4
MCW0038	5	74.6	0.5	0.7	5	16.9
MCW0078	5	104.2	0.8	0.8	5	26.4
MCW0029	5	130.9	0.9	1	5	38.6
LEI0149	5	133.5	0.3	0.9	5	40.9
MCW0081	5	145.2	0.7	0.8	5	45.7
LEI0192	6	0	0.5	0.8	6	2.4
MCW0118	6	0.5	0.6	0.8	6	2.5
MCW0250	6	26.5	1	0.9	6	14.7
ADL0377	6	63.0	0.6	0.8	6	27.3
LEI0196	6	67.8	0.9	0.9	6	28.9
ADL0169	7	0	0.6	0.6	7	37.0
MCW0236	7	37.4	0.9	0.9	7	28.8
ADL0279	7	50.8	0.9	0.9	7	24.5
MCW0120	7	75.9	0.1	0.3	7	11.7
MCW0305	8	0	1	1	8	6.7
ADL0172	8	97.0	0.5	0.4	8	28.2
ADL0278	8	100.0	0.2	0.5	8	29.2
MCW0024	9	0	0.5	0.5	9	9.7
MCW0135	9	19.0	0.2	0.4	9	12.0
ADL0219	9	64.5	0.7	0.8	9	20.5
MCW0134	9	79.1	0.9	0.9	9	22.6
MCW0228	10	0	0.6	0.7	10	1.3
ADL0209	10	33.1	0.8	0.9	10	4.0
MCW0194	10	40.4	0.2	0.8	10	6.5
MCW0067	10	43.8	0.5	0.8	NH‡	
ADL0272	10	48.9	0.4	0.7	10	11.6
ADL0106	10	72.9	0.1	0.4	10	15.3
ADL0158	10	91.2	0.3	0.4	10	17.7
ADL0123	11	0	0.3	0.3	11	5.4
ADL0308	11	44.4	0.1	0.3	11	15.9
ABR0037	11	65.6	0.5	0.6	11	18.9
LEI0099	12	0	0.8	0.8	12	12.1
ADL0044	12	0.4	0.7	0.8	12	12.0
LEI0131	12	16.7	0.9	0.9	NH‡	
ADL0372	12	58.2	0.2	0.3	12	0.7
ROS0325	13	0	0.7	0.8	13	8.5
MCW0213	13	3.2	0.5	0.8	13	1.0
MCW0315	13	20.8	0.9	0.9	13 Random	1.1
ADL0225	13	33.2	0.7	0.8	13	15.7
LEI0066	14	0	0.6	0.6	14	20.3
MCW0296	14	85.5	0.2	0.9	14	3.7
ADL0118	14	86.4	0.9	0.9	14	2.3
LEI0083	15	0	0.9	0.9	15	2.7
ADL0039	15	8.6	0.5	0.8	15	5.6
MCW0211	15	35.9	0.2	0.4	15	10.4
ADL0199	17	0	0.9	0.9	17	10.5
ADL0149	17	35.6	0.4	0.5	17	5.7
MCW0217	18	0	0.4	0.6	18	3.0
ADL0184	18	13.4	0.1	0.7	18	4.8
MCW0119	20 (E47W24)§	0	0.3	0.4	20	0.4
ADL0125	20 (E47W24)§	31.1	0.5	0.6	20	3.0
HCK	20 (E32)§	77.2	0.1	0.5	20	9.7
MC3R	20 (E32)§	90.3	0.9	0.9	20	11.6
BMP7	20 (E32)§	95.4	0.1	0.7	20	11.2
MCW0249	23	0	0.3	0.3	23	4.2
LEI0069	24	0	0.9	0.9	24	4.7
ROS0302	24	22.4	0.4	0.6	UN†	
LEI0074	26	0	0.9	0.9	26	4.2
MCW0069	26	35.1	0.5	0.9	26	1.2
MCW0209	26	38.2	0.9	0.9	26	0.9
MCW0286	26	40.4	0.6	0.9	26	0.7
MCW0076	27	0	0.2	0.5	NH‡	
ADL0376	27	13.2	0.7	0.8	27	2.5
MCW0292	27	22.3	0.6	0.8	NH‡	
MCW0227	28	0	0.5	0.5	Z	19.0
MCW0188	E22C19W28	0	0.7	0.9	NH‡	
ROS0306	E22C19W28	1.8	0.8	0.9	UN†	
GCT0004	E50C23	0	0.3	0.3	E50C23	0.0

Continued

TABLE 1 continued.

Marker	Linkage map				Genome assembly	
	Linkage group	Position (cM)	IC^{mrk}	IC^{pt}	Chromosome	Position (Mb)
ADL0022	Z	0	0.7	0.9	Z	0.1
MCW0331	Z	8.9	0.4	0.8	Z	5.8
ROS0301	Z	15.3	0.8	0.9	Z	13.4
MCW0258	Z	19.0	0.4	0.8	Z	5.2
ADL0273	Z	29.5	0.7	0.8	Z	11.1
LEI0229	Z	42.3	0.6	0.9	Z	16.5
ADL0250	Z	44.4	0.9	0.9	Z	17.6

[1]Markers in bold were assigned to the chicken linkage map in the current study. Map positions are in Kosambi centimorgans (sex-average) relative to the position of the first marker on each chromosome. Chromosome locations in the chicken genome assembly as of February 2004 (http://genome.ucsc.edu) are given for each marker.

*Previously mapped to chromosome 9.

†Unassigned.

‡No hit to the genome assembly as of February, 2004.

§Our data merge linkage group E32 to E47W24 and these linkage groups are both on chromosome 20 in the genome assembly.

PIC were used to evaluate the order of markers along the chromosomes and to estimate the map distance between markers.

Recombination Rates on Macro- and Microchromosomes

The relative recombination rates on macro- and microchromosomes were estimated by comparing genetic and physical distances. Primer sequences were blasted against the genome assembly to retrieve the marker positions. Distances were calculated only for marker pairs in which the genetic map order was in concordance with the assembly. Marker pairs with a genetic distance greater than 50 cM were also excluded.

RESULTS AND DISCUSSION

Linkage Map

A total of 145 microsatellite markers and 3 SNP were typed, and they formed 25 linkage groups in the chicken genome. The total map length, summarizing the intervals flanked by markers, was 2,426.6 cM. In addition, 4 markers did not show linkage to any other marker. The average distance between adjacent markers assigned to linkage

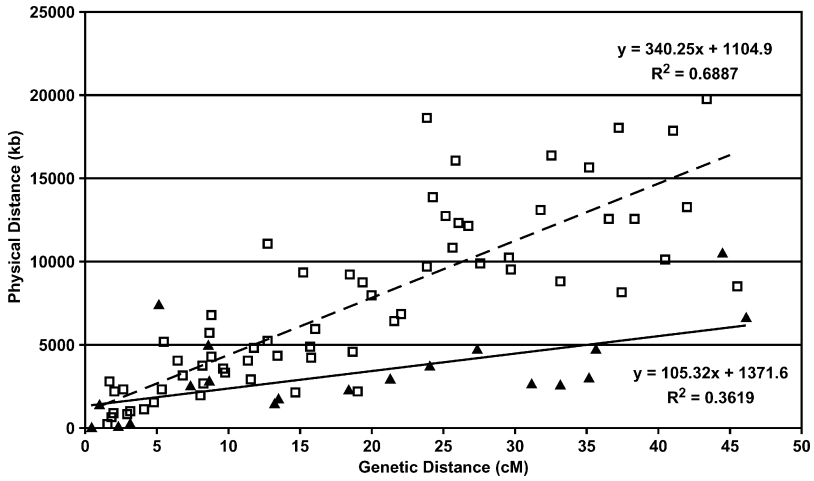

FIGURE 1. Correlation between genetic and physical distances on macrochromosomes (□) and microchromosomes (▲). Primer sequences for genetic markers were blasted against the genome assembly to retrieve the marker positions. Marker pairs with a genetic distance greater than 50 cM were excluded. Linear regression lines for macro- (broken line) and microchromosomes (full line) reveal a 3-fold higher recombination rate on microchromosomes.

groups was 17.0 cM; however, there were 7 gaps greater than 40 cM. Table 1 lists all markers with information content, map position, and the corresponding position in the chicken genome assembly (February 2004). Linkage map assignments for 14 previously unmapped markers (marked in bold in Table 1) were accomplished. As a consequence of the low abundance of microsatellite markers on microchromosomes, many of the microchromosomes had either 0 or only 1 microsatellite marker genotyped.

Four markers (*MCW0169*, *MCW0305*, *LEI0066*, and *MCW0227*) did not show linkage to any other typed marker. Three of these were too far out on a chromosome to show linkage [i.e., *MCW0169* on chicken chromosome 3 (GGA3), *MCW0305* on GGA8, and *LEI0066* on GGA14]. These markers were added to the linkage map according to published maps (Schmid et al., 2000), which is also consistent with the genome assembly. The markers *LEI0134* and *GCT0001* showed linkage to each other [recombination fraction (θ) = 0.05; LOD score (Z) = 73.2], and they showed loose linkage to *ADL0245* on GGA1 (θ = 0.45; Z = 1.5) in accordance with the consensus map. Similarly, *ADL0143* and *ADL0317* showed linkage to each other (θ = 0.11; Z = 83.4) and loose linkage to *ADL0145* on GGA4 (θ = 0.44; Z = 2.1).

The current data provide conclusive evidence that *ADL0132* is located on GGA4 (Z = 152.0; θ = 0.02 to *MCW0091*), which differs from a previous assignment to GGA9 (Cheng et al., 1995). In fact, the chicken genome assembly confirms that *ADL0132* is located on GGA4. The present data showed that the markers *ADL0353* and *ROS0310* were in fact the same marker. Map locations for all other previously mapped markers were in excellent agreement with previously published data.

The linkage data in the current study fit well with the chicken genome assembly of February 2004 with a few exceptions. On chromosome 1 the order of *MCW0168* and *MCW0248* was reversed between the 2 maps, and the map in the present study was supported by a Z score of 2.3. As for the order of *LEI0125* and *LEI0122*, as well as *ADL0143* and *ADL0317* on chromosome 4, the present data may be less reliable because the information content in *LEI0122* was low (0.2) and the LOD supports were only 0.6 and 1.7, respectively. On GGA2 the order of the markers between *LEI0070* and *MCW0311* in the current study was strongly supported with a LOD of 46.0 compared with the order given in the genome sequence and with a Z of 2.0 compared with the second best fit when using the FLIPS option in CRI-MAP. Furthermore, the order of *ROS0325* and *MCW0213* was reversed in our map of GGA13 compared with the genome sequence, and there was strong statistical support for our order (Z = 5.9). Previously, *MCW0227* has been mapped to chromosome 28 (https://acedb.asg.wur.nl). In contrast to the present data, the chicken genome assembly suggests that *MCW0227* is located on the Z chromosome, which seems unlikely because the marker showed an autosomal inheritance pattern. Besides *MCW0227*, there was a discrepancy concerning the order of markers between *MCW0331* and *ADL0273* on the Z chromosome. A Z of 150.6 supports the order in the current study in favor of the order in the genome assembly. On chromosome 20 the order of *MC3R* and *BMP7* differed from the genome sequence. It was not possible to determine the order of the genes with great confidence due to the low information content in *BMP7* (0.1).

TABLE 2. Linkage data for chicken linkage groups E47W24 and E32[1]

Marker 1	Marker 2	θ	Z
MCW0119	*ADL0125*	0.27	20.5
MC3R	*ADL0125*	0.40	5.3
BMP7	*ADL0125*	0.41	1.2
MC3R	*BMP7*	0.06	23.5
MC3R	*HCK*	0.15	10.5

[1]Data are shown for *MCW0119* and *ADL0125* on linkage group E47W24 and *BMP7*, *HCK*, and *MC3R* on linkage group E32. Recombination fractions (θ) and LOD scores (Z) are given for each marker pair with Z > 1.0.

Mapping of MC3R, BMP7, and HCK Merge Linkage Groups E32 and E47W24

Close linkage of *MC3R* to *BMP7* and *HCK* was observed on linkage group E32 (Table 2). The information content in both *BMP7* and *HCK* was low (0.07 and 0.06, respectively); however, the linkage to *MC3R* was highly significant with Z scores of 23.5 and 10.5, respectively. This finding is consistent with Schiöth et al. (2003), who have used the FISH mapping technique to show that *MC3R* and *BMP7* map to the same microchromosome. The *MC3R* also showed linkage to *ADL0125* on linkage group E47W24 (θ = 0.40; Z = 5.3), demonstrating that the 2 linkage groups E32 and E47W24 are located on the same chromosome. This result has now been confirmed due to the release of the chicken genome assembly, which showed that all of the above-mentioned loci map to GGA20.

Information Content

Average information content was 0.55 on the individual marker basis. However, information content at marker positions was 0.72 when data from flanking markers were taken into account. For 60 markers the information content was less than 0.5, but this number was reduced to 26 when adjacent marker information was included. The informativeness of the 647 initially tested markers was generally lower in this intercross than that found in an intercross between the red jungle fowl and White Leghorn chickens (Kerje et al., 2003). A reasonable explanation for this finding is that the 2 parental chicken lines used in the experiment stem from the same chicken population prior to 41 generations of strong selection for high and low body weight and, therefore, may often share the same alleles except for those regions of the genome that has responded to selection. The 2 lines have been kept at a population size sufficiently large enough to avoid exten-

sive inbreeding. The accumulated inbreeding coefficient was estimated at 0.53 in generation 41.

Recombination Rates on Macro- and Microchromosomes Differ Significantly

A highly significant 3-fold higher recombination rate was observed on microchromosomes than on macrochromosomes (Figure 1). The linear regression analysis indicated that 1 cM corresponds to ~105 kb on microchromosomes compared with ~340 kb on macrochromosomes. However, the estimate for microchromosomes is a bit uncertain because several microchromosomes were not included in the analysis due to a lack of markers. The current results are in good agreement with previous studies that have indicated a recombination rate of on average 396 kb/cM on macrochromosomes and 150 to 250 kb/cM for microchromosomes (Schmid et al., 2000).

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II

The twofold difference in adult size between the red junglefowl and White Leghorn chickens is largely explained by a limited number of QTLs

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Summary

A large intercross between the domestic White Leghorn chicken and the wild ancestor, the red junglefowl, has been used in a Quantitative Trait Loci (QTL) study of growth and egg production. The linkage map based on 105 marker loci was in good agreement with the chicken consensus map. The growth of the 851 F_2 individuals was lower than both parental lines prior to 46 days of age and intermediate to the two parental lines thereafter. The QTL analysis of growth traits revealed 13 loci that showed genome-wide significance. The four major growth QTLs explained 50 and 80% of the difference in adult body weight between the founder populations for females and males, respectively. A major QTL for growth, located on chromosome 1 appears to have pleiotropic effects on feed consumption, egg production and behaviour. There was a strong positive correlation between adult body weight and average egg weight. However, three QTLs affecting average egg weight but not body weight were identified. An interesting observation was that the estimated effects for the four major growth QTLs all indicated a codominant inheritance.

Keywords additive effects, chicken, egg production, growth, Quantitative Trait Locus.

Introduction

Domestic animals provide unique opportunities to study the genetic basis for phenotypic diversity and are excellent models for evolution by natural selection (Andersson 2001). We have generated a resource pedigree for mapping Quantitative Trait Loci (QTLs) by crossing the red junglefowl (*Gallus gallus* spp.) with White Leghorn chickens. The red junglefowl is the wild ancestor of the domestic chicken and the process of chicken domestication is believed to have started well over 8000 years ago in South-east Asia (Yamada 1988; Fumihito *et al.* 1994). Initially the chicken was used as a sacrificial or religious bird, or for cockfighting. It was the Romans who developed its potential as an agricultural animal, creating specialized breeds, including dual-purpose breeds and productive layers. With the decline of the Roman Empire the poultry industry collapsed and very little systematic selection was practiced for many centuries, with the exception of birds for cockfighting. The Leghorn type chicken is derived from the mediterranean type of chicken and was developed during the nineteenth century. The White Leghorn is a light, egg-laying breed that has been selected for efficiency – maximum output of eggs for minimum food intake. Despite this, the White Leghorn is about twice as large as the red junglefowl, and this marked phenotypic difference was utilized in the present study. The red junglefowl and White Leghorn chickens also differ markedly for a number of other traits including plumage colour, egg weight, egg production, age of sexual maturity and, as recently demonstrated, behaviour (Schütz *et al.* 2001, 2002; Schütz & Jensen 2001). A red junglefowl by White Leghorn backcross, established by others, has been widely used for chicken genome mapping but not for QTL mapping (Crittenden *et al.* 1993). Thus, although there are a number of previous QTL studies in the chicken (Dunnington *et al.* 1992; Plotsky *et al.* 1993; Vallejo *et al.* 1998; Van Kaam *et al.* 1999a, b; Yonash *et al.* 1999, 2001; Tatsuda & Fujinaka 2001a, b; Ikeobi *et al.* 2002; Sewalem *et al.* 2002) this is the first study testing for QTL differences between the red junglefowl and a domestic breed.

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In this paper we report a genome scan for QTLs affecting growth, body size and egg production based on data on more than 800 F_2 animals scored for more than 100 genetic markers.

Material and Methods

Animals

A three-generation resource pedigree was generated after mating one red junglefowl male with three White Leghorn females (Schütz *et al.* 2002). The red junglefowl male was obtained from a Swedish zoo, and originated from a relatively closed European zoo population, originally obtained from Thailand. The particular line of White Leghorn used in this cross (SLU13) has been developed at the Swedish University of Agricultural Sciences (Liljedahl *et al.* 1979). Four F_1 males and 37 F_1 females were intercrossed and 851 F_2 animals have been used for the QTL study. Animals were kept at the research facilities, Swedish University of Agricultural Sciences, Skara where all phenotype recordings were performed. The F_2 animals were raised in six batches comprising about 150 birds each.

Phenotypic traits

All F_2 birds were weighed at 1, 8, 46, 112 and 200 days to obtain growth rates. Average egg weight and total egg production were measured individually at 29 weeks of age by collecting eggs for 1 week.

DNA isolation, marker selection and genotyping

Blood samples were collected from all F_2 individuals, their parents (F_1) and grandparents (F_0). Seven microlitres of blood were used for DNA isolation using the DNeasy^{TM}96 Tissue Kit for mouse tails (Qiagen, Valencia, CA, USA) with some minor modifications.

A total of 189 previously described microsatellite markers were initially tested on a limited number of animals to select the most informative ones to be used in this study. A set of 105 markers was selected for the genome scan. The information content for each marker was calculated using the web based QTL Express software (Seaton *et al.* 2002; http://qtl.cap.edu.ac.uk/). Primer details for microsatellite markers can be found at http://poultry.mph.msu.edu or http://www.thearkdb.org/.

Polymerase chain reaction (PCR) amplifications of the microsatellite markers were carried out using fluorescently labelled primers. Polymerase chain reactions were performed in a total volume of 5 μl containing 1× PCR Buffer II (Applied Biosystems, Foster City, CA, USA), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 1–5 pmol of each primer, 0.25 U of AmpliTaq Gold^{TM} DNA polymerase (Applied Biosystems) and 20–50 ng of genomic DNA. The PCR reaction was initiated with an incubation for 5 min at 95 °C to activate the polymerase, followed by a touchdown PCR cycle starting with annealing for 30 s at 65 °C and decreasing by 1 °C per cycle to 52 °C. Forty cycles of PCR were performed with annealing at 52 °C, denaturation for 45 s at 95 °C and extension for 30 s at 72 °C. The last cycle included an extension step for 5 min at 72 °C. The PCR products were denatured 2 min before electrophoresis in 4% polyacrylamide gels using an ABI377 sequencer (Applied Biosystems) or a MegaBACE capillary instrument (Amersham Biosciences, Uppsala, Sweden). The results were analysed with the Genescan and Genotyper software (Applied Biosystems) or Genetic Profiler (Amersham Biosciences).

Five additional loci were included in the genome scan. The dominant white locus (*I*) for plumage colour was scored as a single dominant trait, for which the White Leghorn (*I*/*I*) and the red junglefowl (*i*/*i*) are fixed for different alleles. The two populations are also fixed for alternate alleles at the melanocortin-1 receptor (*MC1R*) locus controlling black or wild type plumage colour; the presumed causative mutation was scored using pyrosequencing (Kerje *et al.* 2003). Highly informative PCR-RFLPs representing the loci for the melanocortin-3 receptor (*MC3R*; S. Jiang, S. Kerje & L. Andersson, unpublished data), melanocortin-4 receptor (*MC4R*; S. Jiang, S. Kerje & L. Andersson, unpublished data) and the KIT receptor (*KIT*; described below) were also used.

A 570 bp fragment from the *KIT* gene was amplified using the chKITfwd (5′-TTACATAGACCCAACGCAACT–3′) and chKITrev (5′-TAGTGCAAGCTCCAAGTAGAT– 3′) primers designed from the cDNA sequence in GenBank (D13225). The PCR contained 1× PCR buffer II (Applied Biosystems), 1.87 mM $MgCl_2$, 300 μM of each dNTP, 20 pmol of each primer, 1 U of AmpliTaq Gold^{TM} DNA polymerase (Applied Biosystems) and about 100 ng DNA in a total volume of 20 μl. The following PCR profile was used in a PTC-200 thermal cycler (MJ Research, Inc., Waltham, MA, USA), 5 min at 94 °C, 35 cycles with 45 s at 94 °C, 30 s at 53 °C, 1 min at 72 °C and finally 5 min at 72 °C. The PCR product was purified using the QIAquick® PCR Purification Kit (Qiagen) and sequenced from both ends with BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems). A sequence comparison revealed a single nucleotide polymorphism, where the White Leghorn sequence had a *Taq*I recognition site, which was utilized for genotyping using a PCR-RFLP assay. For the restriction enzyme reaction, 15 μl of the PCR reaction (generated as described above) was digested with 1.5 U *Taq*I enzyme (New England BioLabs, Inc., Beverly, MA, USA) for 1 h at 65 °C in 1× *Taq*I buffer (New England BioLabs). The alleles were scored after electrophoresis in an 1.5% agarose gel (Nusieve:Seakem, 1:1).

Statistical analysis

Linkage maps for 25 autosomal linkage groups were generated using the CRI-MAP software (Green *et al.* 1990). The

functions FLIPS and FIXED were used to evaluate the order of markers along the chromosomes and to estimate the map distance between markers. The sex-specific recombination rates were estimated using CRI-MAP and the statistical evaluation was done with a likelihood ratio test (Ott 1985); this test statistic is expected to follow a χ^2 distribution with degrees of freedom equal to the number of marker intervals tested.

The software used for QTL mapping was developed for improved computational efficiency. This has been achieved by utilization of parallel computing, supercomputers and the application of new efficient numerical algorithms (Carlborg *et al.* 2001; Carlborg 2002; Ljungberg *et al.* 2003). The method used for QTL mapping is based on the ordinary least-squares based method for mapping QTL in outbred line crosses described by Haley *et al.* (1994). Marker genotypes were used to estimate the probabilities of breed origin of each gamete at 1 cM intervals throughout the genome for each F_2 individual. These probabilities were used to calculate additive and dominance coefficients for a putative QTL at each position under the assumption that the QTL was fixed for alternative alleles in the two breeds. The trait values were then regressed onto these coefficients in intervals of 1 cM. The additive and dominance regression indicator variables for the most significant single QTL in this scan were added as cofactors to the model used for the scan and a new genome scan was performed using the updated model. Adding the previously detected QTL to the model decreases the error variance, which will increase the power to detect QTLs with smaller genetic effects. This procedure was repeated until no additional significant QTL was detected.

The least-squares regression model used for QTL analysis included the fixed effects of sex and batch along with additive and dominance coefficients for the putative QTL for all traits. There was a highly significant correlation between body weight at 200 days and egg weight. Therefore, body weight at 200 days was included as a covariate in the QTL analysis to allow us to detect differences in egg weight at a fixed weight.

Statistical significance was assessed in each successive step in the QTL mapping procedure by randomization testing using 1000 permutations of data (Churchill & Doerge 1994). Genome-wide thresholds (1 and 5%) for significant QTLs and a 20% genome-wide significance threshold for suggestive QTLs were applied. Thus, we have used a more stringent threshold for suggestive QTLs compared with the commonly used threshold that is expected to give one false positive QTL per genome scan (Lander & Kruglyak 1995). The two randomization testing thresholds were fairly constant throughout the study.

Correlation coefficients and the proportion of residual variances explained by the detected QTLs were calculated using the SAS software (SAS 1990).

Results

Descriptive statistics

We analyzed body weights, growth and egg production of 767-814 F_2 chickens. Corresponding estimates were measured for the parental lines in the same environment and with the same feeding regime but not in parallel during the same time period. The body weight for the parental red junglefowl and White Leghorn populations and for the F_2 chickens were measured at 1, 8, 46, 112 and 200 days of age, and between these ages growth rates were calculated. The phenotypic means, standard errors of the means and standard deviations for weight at hatch, the growth traits and egg production traits are given in Table 1. The growth of the F_2 chickens was lower than the parental lines prior to 46 days of age, whereas it was intermediate to the two parental lines for growth after 46 days of age.

Linkage map

The linkage analysis was based on 105 loci, including 100 microsatellites, four SNPs and one phenotypic trait, representing 25 of 39 chromosomes in the chicken genome leaving 14 microchromosomes uncovered. The average information content for all markers was 0.77 (Table 2). The sex-averaged map spanned 2552 cM and the average marker spacing was 24.3 cM. The marker order

	Red junglefowl $n = 20$		White Leghorn $n = 31$		F_2 progeny $n = 767$–814	
Trait	Mean ± SEM	SD	Mean ± SEM	SD	Mean ± SEM	SD
Body weight 1 day (g)	26.5 ± 0.6	2.7	37.6 ± 0.9	4.8	36.9 ± 0.1	3.9
Growth 1–8 days (g)	38.5 ± 2.5	11.0	46.1 ± 1.6	9.0	10.4 ± 0.2	4.9
Growth 8–46 days (g)	316.4 ± 15.9	71.1	505.4 ± 12.2	67.7	269.6 ± 1.9	52.8
Growth 46–112 days (g)	414.6 ± 30.5	136.5	758.7 ± 43.6	242.8	607.9 ± 5.4	153.5
Growth 112–200 days (g)	147.3 ± 14.8	66.0	426.3 ± 18.5	102.8	353.8 ± 4.3	121.9
Egg weight (g)	23.0 ± 6.2	19.8	57.5 ± 3.8	15.2	43.2 ± 0.6	11.1
Total egg weight (g)[1]	97.3 ± 30.5	96.6	367.1 ± 27.4	109.6	221.9 ± 3.9	77.8

[1]Produced during 1 week.

Table 1 Weight at hatch, four growth rates and egg production measured in red junglefowl, White Leghorn and red junglefowl × White Leghorn F_2 chickens. Mean, standard errors of the mean (SEM) and standard deviations (SD) are provided.

Table 2 Genetic markers used for QTL mapping in a red junglefowl × White Leghorn intercross and information content (IC) for each marker. Distances are in Kosambi cM relative to the position of the first marker on each chromosome.

Marker	Chromosome/linkage group	Position sex average map	IC
ADL160	1	0	0.88
LEI209	1	27.7	0.71
MCW010	1	35.3	0.73
ADL019	1	91.3	0.87
LEI146	1	124.3	0.87
MCW018	1	154.2	0.91
LEI071	1	189.7	0.97
LEI101	1	209.3	0.89
MCW068	1	233	0.92
LEI088	1	258.8	0.83
LEI139	1	337.4	0.89
LEI107	1	372.3	0.91
LEI246	1	407.9	0.50
ADL328	1	425.9	0.87
LEI134	1	475.4	0.30
ADL228	2	0	0.87
MCW247	2	77.8	0.87
MCW063	2	125.6	0.77
ADL257	2	157.9	0.82
MCW062	2	168.4	0.89
MCW042	2	229.3	0.95
MC4R	2	242.6	0.89
MCW087	2	259.2	0.87
LEI147	2	280.3	0.85
MCW264	2	316.2	0.66
MCW166	2	335.5	0.94
LEI070	2	358.2	0.95
MCW176	2	362.9	0.78
MCW073	2	448.7	0.77
MCW157	2	467.6	0.83
MCW261	3	0	0.81
MCW169	3	30.3	0.40
HUJ006	3	101.9	0.80
LEI161	3	131.1	0.90
LEI115	3	161.8	0.94
ADL371	3	167.4	0.95
MCW126	3	233.9	0.66
LEI265	3	254.1	0.90
ADL237	3	273.4	0.87
ADL255	4	0	0.06
ADL145	4	70	0.93
MCW005	4	81.7	0.92
ADL266	4	113.2	0.92
LEI094	4	128.3	0.89
KIT	4	164.1	0.43
MCW122	4	183	0.78
LEI073	4	208.8	0.69
LEI082	5	0	0.83
MCW038	5	38.9	0.38
MCW029	5	82.5	0.92
MCW081	5	97.4	0.87
ADL323	6	0	0.86
ADL036	6	49.8	0.66
LEI097	6	71.1	0.93
MCW250	6	81.6	0.87
LEI192	6	117.2	0.93
ADL169	7	0	0.91
MCW236	7	34.9	0.80
MCW133	7	65.3	0.91
LEI064	7	165.3	0.52
ADL278	8	0	0.87
ADL154	8	60.8	0.88
ADL258	8	75.7	0.83
ADL191	9	0	0.63
MCW135	9	16.8	0.84
ADL136	9	42.9	0.39
MCW228	10	0	0.83
ADL209	10	27.5	0.47
ADL038	10	45.3	0.64
ADL158	10	99.9	0.53
LEI110	11	0	0.19
ADL210	11	47.6	0.93
ADL308	11	70.2	0.88
MC1R	11	93.2	0.91
ADL044	12	0	0.90
ADL372	12	66.1	0.51
MCW322	13	0	0.91
MCW213	13	26	0.85
ADL118	14	0	0.86
LEI098	14	38.1	0.86
MCW211	15	0	0.71
LEI120	15	52	0.90
ADL293	17	0	0.49
ADL290	18	0	0.84
ADL304	18	28.4	0.39
MCW256	19	0	0.55
MCW287	19	22.5	0.83
LEI090	23	0	0.96
MCW165	23	81.2	0.91
MCW069	26	0	0.91
MCW300	27	0	0.37
MCW328	27	27.3	0.89
ADL284	28	0	0.27
ADL299	28	34.8	0.90
I	E22C19W28	0	0.44
MCW317	E22C19W28	22.9	0.21
LEI080	E47W24	0	0.89
GCT004	E50C23	0	0.69
MC3R	UN		0.95
MCW055	Z	0	0.88
ADL273	Z	40.3	0.91
MCW241	Z	50.5	0.94
LEI229	Z	56.7	0.93
LEI121	Z	77.4	0.91
LEI075	Z	106.3	0.89

UN, unassigned.

Table 3 Summary statistics of the chicken linkage map based on a red junglefowl/White Leghorn intercross.

Chromosome/linkage group	Number of loci	Map length (Kosambi cM)			Sex differences χ^2_{df}
		Average	Female	Male	
1	15	475.4	471.5	489.4	35.9_{14}**
2	15	467.6	520.4	434.5	39.2_{14}***
3	9	273.4	269.9	277.7	4.0_{8}
4	8	138.8	154.2	125.6	43.6_{7}***
5	4	97.4	103.9	90.5	7.0_{3}
6	5	117.2	112.9	121.9	13.0_{4}*
7	4	165.3	163.9	168.0	8.9_{3}*
8	3	75.7	98.1	63.1	4.8_{2}
9	3	42.9	43.1	45.9	4.7_{2}
10	4	99.9	105.2	89.5	5.0_{3}
11	4	93.2	80.3	104.8	11.9_{3}**
12	2	66.1	100.0	52.8	3.1_{1}
13	2	26.0	31.3	21.3	8.2_{1}**
14	2	38.1	36.7	39.6	0.2_{1}
15	2	52.0	55.2	48.8	0.7_{1}
18	2	28.4	28.8	27.8	0_{1}
19	2	22.5	25.6	19.5	1.8_{1}
23	2	81.2	82.7	79.7	0_{1}
27	2	27.3	19.6	36.7	2.1_{1}
28	2	34.8	34.8	34.8	0_{1}
E22C19W28	2	22.9	22.9	0	0_{1}
Z	6	–	–	106.3	
Total	94	2446.1[1]	2561.0[1]	2371.9[1]	194.1_{73}***

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
[1]Includes only autosomes.

corresponds very well with the chicken consensus map (Groenen *et al.* 2000; Schmid *et al.* 2000) but with one exception. *MCW176* is found on chromosome 6 in the consensus map but we mapped it to chromosome 2. This assignment was supported by highly significant lod scores to several markers (e.g. lod score = 51.3, recombination fraction = 0.05 against *LEI070*).

The intercross design and the large size of this pedigree allowed us to test for sex differences in recombination rates. We found significant sex differences for seven linkage groups (Table 3). However, there was no clear overall trend because the male map was longer in four cases and the female map in the other three. The total map length for autosomes was marginally longer (+8%) in females (2561 cM) than in males (2372 cM).

QTL analysis of growth and body weight

Nine measurements of body weight and growth were tested. We did not find any QTL for weight at hatch and this was not unexpected as this trait has a very strong maternal component. Among the other eight traits, 38 QTL tests were declared significant at least at the 20% suggestive level (Table 4); QTL graphs for the four major loci affecting adult body weight are shown in Fig. 1. These represented a minimum of 14 QTLs, designated *Growth1* to *Growth14*, when adopting a conservative interpretation of the number of QTLs. This means that we did not infer more than a single QTL for a given trait in a chromosome region unless the two estimated QTL positions differed by a considerable recombination distance, >30 cM. As many as 13 of these QTLs were significant at the 5% level for at least one growth or body weight trait. There was also a very clear trend that QTL alleles inherited from the red junglefowl were associated with a lower growth rate and smaller body size as expected from the difference between populations (Table 4). There were three exceptions to this rule. *Growth9* on chromosome 7 was significant for only one trait, body weight 112 days, and the red junglefowl allele was associated with slightly higher body weight but the major effect of this locus appears to be overdominance i.e. a superior growth of the heterozygote. *Growth10* on chromosome 8 affected early growth between day 1 and 8, and the red junglefowl allele increased the growth rate slightly. Finally, the red junglefowl allele at *Growth14* on the Z chromosome was associated with higher growth but the effect was restricted to female growth (data not shown). This may reflect a sex-specific effect of this QTL or a recessive inheritance of the low growth allele from White Leghorn as the F_2 males in this cross were Z^{rjf}/Z^{wl} or Z^{rjf}/Z^{rjf} whereas F_2

Table 4 Quantitative Trait Loci (QTL) for growth (GR), body weight (BW) and egg weight (EW) detected in a red junglefowl/White Leghorn intercross. Test statistics, estimated QTL effects, % of residual F_2 variance explained by each QTL and covariates used in the QTL analysis are given.

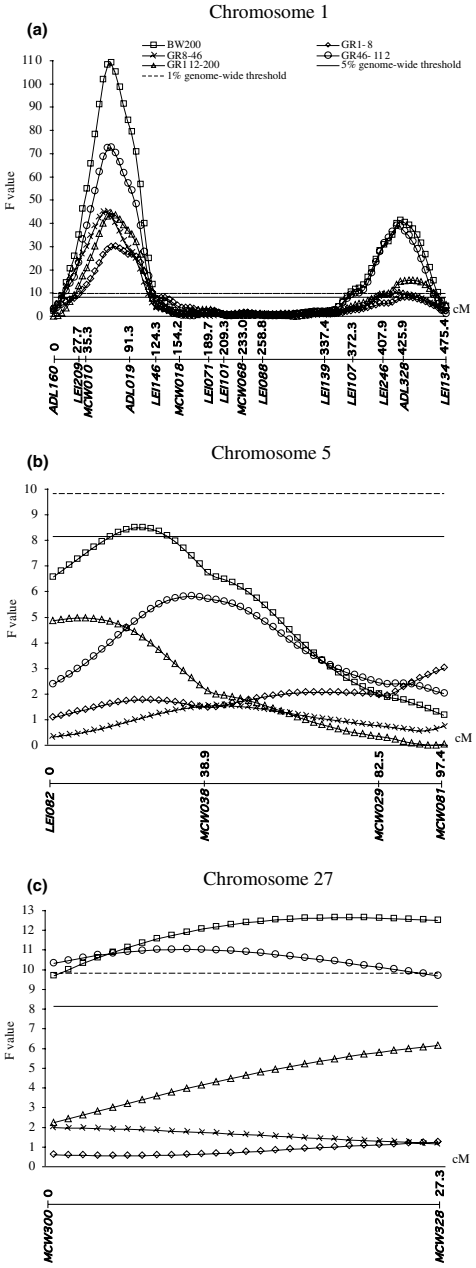
QTL	Chr.	Position, cM	Trait (g)	F-value[1]	Additive effect ± SE[2]	Dominance effect ± SE[2]	% variance[3]	Covariates
G1	1	71	BW8	22.4**	−2.6 ± 0.4	1.1 ± 0.8	5.5	
		64	BW46	50.0**	−35.9 ± 3.6	5.9 ± 7.9	11.1	
		67	BW112	81.3**	−114.7 ± 8.9	−1.0 ± 19.4	16.8	
		68	BW200	109.4**	−173.2 ± 11.6	9.6 ± 25.0	21.6	
		73	GR1-8	30.0**	−2.5 ± 0.3	0.3 ± 64.0	7.3	
		63	GR8-46	45.0**	−32.4 ± 3.4	7.7 ± 7.5	10.6	
		68	GR46-112	72.9**	−78.3 ± 6.4	−0.3 ± 13.9	15.4	
		70	GR112-200	44.2**	−59.7 ± 6.3	0.3 ± 13.3	10.0	
		58	Total EW	15.3**	−26.4 ± 5.6	41.5 ± 12.4	7.8	
		61	Aver. EW	57.8**	−5.1 ± 0.5	2.8 ± 1.1	24.1	
		59	Aver. EW	19.4**	−2.8 ± 0.4	1.6 ± 0.9	9.6	BW200
G2	1	399	BW8	6.6†	−1.1 ± 0.4	1.4 ± 0.7	1.7	
		419	BW46	14.8**	−14.8 ± 2.7	4.1 ± 4.2	3.6	G1
		418	BW112	39.7**	−60.9 ± 6.8	−1.3 ± 10.7	9.0	G1
		420	BW200	50.6**	−86.7 ± 8.6	7.9 ± 13.1	11.3	G1
		426	GR1-8	7.1†	−0.9 ± 0.2	−0.2 ± 0.3	1.9	
		417	GR8-46	11.1**	−12.4 ± 2.7	6.0 ± 4.3	2.8	G1
		416	GR46-112	44.4**	−47.6 ± 5.1	−4.4 ± 8.1	10.0	G1
		431	GR112-200	15.9**	−27.5 ± 4.9	6.1 ± 7.4	3.9	G1
G3	2	411	BW200	8.4*	−49.4 ± 13.2	71.9 ± 38.3	2.1	G1, G2, G6, G8, G13
G4	3	50	BW8	8.8*	−0.7 ± 0.5	−5.1 ± 1.3	2.3	G1
G5	3	117	BW46	6.7†	−8.3 ± 2.8	−12.2 ± 4.7	1.7	
		112	GR1-8	9.4*	−1.0 ± 0.3	−1.2 ± 0.5	2.4	G1, G10
E1	3	162	Aver. EW	7.7†	0.9 ± 0.2	0.5 ± 0.4	4.1	BW200
G6	3	208	BW112	7.1†	−29.7 ± 10.3	84.9 ± 29.0	1.7	
		201	BW200	8.3*	−37.9 ± 12.5	107.5 ± 33.6	2.1	G1, G2, G8, G13
G7	4	122	BW112	7.9†	−20.0 ± 5.9	22.6 ± 9.2	1.9	
		150	GR46-112	6.8†	−21.3 ± 5.7	4.8 ± 11.2	1.7	
		137	Total EW	7.2†	−17.3 ± 4.5	−1.2 ± 7.4	3.8	
G8	5	21	BW200	8.5*	−44.1 ± 10.6	13.1 ± 21.6	2.1	G1, G2, G13
G9	7	145	BW112	8.4*	28.8 ± 11.0	104.2 ± 31.6	2.1	G1, G2, G6, G13
G10	8	64	BW8	8.0*	0.8 ± 0.3	1.3 ± 0.4	2.1	G1, G4
		69	GR1-8	8.6*	0.9 ± 0.2	−0.8 ± 0.4	2.5	G1
G11	11	92	BW8	7.5†	−1.1 ± 0.3	0.2 ± 0.4	1.9	
		84	BW46	11.7**	−12.8 ± 2.6	3.5 ± 4.2	2.8	G1, G2
		60	GR8-46	9.8**	−10.8 ± 2.4	−2.6 ± 4.1	2.5	G1, G2
G12	12	59	BW46	6.5†	−11.9 ± 3.2	−2.9 ± 6.1	1.6	
		65	BW112	8.8*	−30.6 ± 7.1	−3.4 ± 11.9	2.2	G1, G2, G13
E2	14	14	Aver. EW	11.7**	1.6 ± 0.3	−0.1 ± 0.6	6.1	G1, E3, BW200
E3	23	72	Aver. EW	11.4**	−1.5 ± 0.3	1.4 ± 0.6	5.9	G1, BW200
G13	27	7	BW112	11.0**	−37.0 ± 7.8	−3.6 ± 14.3	2.7	G1, G2
		20	BW200	12.6**	−41.8 ± 8.3	−5.7 ± 13.6	3.1	G1, G2
		9	GR46-112	11.0**	−25.7 ± 5.6	−7.8 ± 10.2	2.7	G1, G2
G14	Z	22	BW200	9.3*	31.5 ± 7.2	−3.6 ± 7.1	2.3	G1, G2, G3, G6, G8, G13
		22	GR112-200	8.9*	18.3 ± 4.3	2.1 ± 4.3	2.2	G1, G2

[1]*F* statistic for the QTL at this genomic location and significance level; **F*-value above the empirical 5% genome-wide significance threshold, varying between 7.9 and 8.6 for different traits; ***F*-value above the empirical 1% genome-wide significance threshold, varying between 9.5 and 10.8 for different traits; †*F*-value above the empirical 20% genome-wide significance threshold at 6.5.

[2]The additive effect (a) and the dominance effect (d) were defined as deviation of animals homozygous for the red junglefowl allele or heterozygous, respectively, from the mean of the two homozygotes. Standard errors (SE) are also given.

[3]Percentage residual variance explained by the QTL.

G1–G14, *Growth1* to *Growth14*; E1–E3, *Eggweight1* to *Eggweight3*; Aver. EW, average weight of eggs produced during 1 week; Total EW, total egg weight during 1 week.



females had the genotype Z^{rjf}/W^{wl} or Z^{wl}/W^{wl} as a result of the design of this intercross (rjf = red junglefowl; wl = White Leghorn).

The two major QTLs for growth were both located on chromosome 1 at around positions 68 and 416 cM (Table 4 and Fig. 1a). *Growth1* (at 68 cM) did not affect weight at hatch but had a large effect on growth from the first week of age and during the entire growth period. This locus on its own explained more than 20% of the residual phenotypic variance for adult body weight and explained about 35% of the difference in adult size between the two populations. Does this very large QTL effect represent a single QTL or a cluster of linked QTLs in this part of the chromosome? To assess this important question we included *Growth1* as a cofactor with the estimated additive effect as given in Table 4. The QTL graph for this region became completely flat showing that recombination is not able to break apart this QTL peak (data not shown). Thus, we conclude that *Growth1* behaves as a single locus that may contain one or several linked causative genes.

The QTLs detected in this study explain a large proportion of the difference in adult body size between the two founder populations. We estimated the individual effects as well as the combined effects of the four major QTLs by including all four loci simultaneously in a least-square analysis (Table 5). The four major QTLs for this trait explain 31% of the residual variance in the F_2 generation and two thirds of the difference between populations in adult body weight (sex-average). An interesting finding was that these four QTLs all show a codominant inheritance as no dominance effect was observed. There is a marked sex-difference in growth in chickens so we also estimated the sex-specific effects of these QTLs. Although we observed the same trend in both sexes it is clear that these QTLs have a more pronounced effect on male growth. There was in fact a significant interaction between the action of *Growth1* and sex ($F_{1,796} = 22.7$, $P < 0.0001$), and between *Growth2* and sex ($F_{1,796} = 7.3$, $P < 0.007$) The four QTLs explained about 80% of the difference between the founder populations for male growth but only about 50% for female growth. The lack of dominance is less clear in the sex-specific estimates but these are also more uncertain as each estimate is based on only 50% of the material.

The QTL analysis has been carried out with a model assuming that the founder populations are fixed for different QTL alleles. The power of QTL detection is reduced and the estimated QTL effects are biased downwards if this assumption is not met. Therefore, we decided to investigate

Figure 1 Test statistic curves for the four major QTLs affecting adult body weight in a red junglefowl/White Leghorn intercross. (a) *Growth1* and 2 on chromosome 1. (b) *Growth8* on chromosome 5. (c) *Growth13* on chromosome 27. The graph represents the test for a single QTL at a given position along the chromosome and the marker map (with the distances between markers in Kosambi cM) is given on the X-axis. The horizontal line shows the 1 and 5% threshold for genome-wide significance.

Table 5 Estimated additive (a) and dominance (d) effects on adult body weight of four major growth Quantitative Trait Loci (QTLs) in comparison with body weight in the parental red junglefowl and White Leghorn populations.

	Sex-average		Males		Females	
Body weight in parentals (in grams)						
Red junglefowl	960		1120		800	
White Leghorn	1870		2110		1630	
Difference	−910		−990		−830	
Effects of QTLs (in grams)						
Locus	2a[1]	d	2a[1]	d	2a[1]	d
Growth1	−306	5	−400	−22	−198	45
Growth2	−166	11	−210	6	−128	6
Growth13	−92	6	−98	43	−74	−29
Growth8	−54	−1	−76	20	−24	−42
Sum	−618		−784		−424	
Percentage residual variance	31.0		38.6		17.1	
Percentage population difference	67.9		79.2		51.1	

[1]The additive effect represents by definition half the estimated phenotypic difference between the two homozygotes and therefore we provide here the estimate for 2a.

this assumption for the four major QTLs for adult body weight by a heterogeneity test among the four large F_1 half-sib families present in this material. There was no significant heterogeneity for *Growth1*, *Growth8* or *Growth13*, but there was a highly significant heterogeneity for *Growth2* ($F_{3,785} = 5.74$, $P = 0.0007$). The results showed that the estimated additive effect of *Growth2* was only -31.6 ± 15.8 for sire 1008 whereas the corresponding estimates for the other three sires were in the range -94.5 ± 20.0 to -119.5 ± 18.1. Thus, sire 1008 may be homozygous at *Growth2* but heterozygous for a linked minor QTL or there may be three alleles segregating at this QTL. The information about this heterogeneity among sires is very important for future attempts to identify the causative gene(s) for this major QTL.

QTL analysis of egg production

The average egg weight showed a strong positive correlation with adult body weight ($r = 0.62$, $P < 0.0001$). The following linear regression between average egg weight (EW in grams) and body weight at 200 days (BW200 in grams) were estimated in the F_2 population: EW = 21.9 + (0.02 × BW200). This means that the larger adult body size in White Leghorn females (+∼800 g) should explain about 50% of the difference in average egg weight between the two populations (Table 1).

As shown in Table 4, the *Growth1* QTL has a huge effect also on the average egg weight. About half of the effect can be explained due to the effect on adult body size but the QTL analysis including body weight as a covariate shows that *Growth1* also has a direct effect on the size of the eggs. The additive effect of this QTL explains about 30% of the difference in average egg weight between the two populations.

Three additional QTLs for average egg weight were detected using a model including body weight as a covariate. These are located on chromosomes 3, 11 and 14, and they were designated *Eggweight1–3* as they were not colocalized with any growth QTL (Table 4). Two of these QTLs showed the expected trend of an association between the red junglefowl allele and smaller eggs whereas *Eggweight2* showed the opposite effect.

Only two QTLs for total egg weight during 1 week were detected and they were both colocalized with two growth QTLs, *Growth1* and *7*. The QTL effect on total egg weight disappeared when body weight was included in the model.

Discussion

A common problem in genetic studies of multifactorial traits is a low statistical power, caused by the combination of limited sample sizes and the rather small effect of each locus. The consequence of this is that reproducibility is poor and the estimated effects of detected QTLs are uncertain and often inflated (Mackinnon & Georges 1992; Goring *et al.* 2001). We generated a large F_2 generation of more than 800 progeny in an attempt to obtain a high statistical power for QTL detection. The results imply that we in fact have achieved this for growth, in particular late growth, because many QTLs segregating in this cross appear to have a sufficiently large effect to be detected in a QTL experiment of this size. This is evident from the fact that 13 of 14 QTLs that were significant at the 20% genome-wide level also were significant at the 5% level, and that the QTLs explain a large part of the difference in adult body weight between the parental populations. This high statistical power in QTL detection allows us to get some insight into the genetic background of growth and to the effects of the QTLs. The classical infinitesimal model for inheritance of multifactorial trait involves an infinite number of loci each with an infinitesimal small effect (Lynch & Walsh 1998). This is obviously an unrealistic theoretical model that has been useful for the development of quantitative genetics theory and its practical application. The ancestor of the domesticated

White Leghorn diverged from the red junglefowl thousands of years ago and our results demonstrate that the twofold difference in adult body weight between these populations is largely explained by a limited number of QTLs with large and moderate effects. We can refute the possibility that the difference is explained by hundreds of QTL each with a very small effect. The QTLs detected in this study do not explain the entire difference in growth but it should be noted that our genome scan is not complete as we are lacking markers on several microchromosomes and there are also some regions on macrochromosomes with poor coverage that may harbour additional QTLs with large or moderate effects. Furthermore, in another paper based on the same material we report that epistasis between QTLs plays a significant role for early growth (Carlborg *et al.* 2003).

An interesting observation was that several of the major QTLs show large additive effects but no significant dominance effects, which means that the heterozygotes have an intermediate phenotype. This is in contrast to the great majority of trait loci with a monogenic inheritance that so far have been studied at the molecular level. A search of the Mouse Genome Informatics database (http://www.informatics.jax.org/; June 2002) with the inheritance mode 'dominant', 'recessive', and 'codominant' gave 367, 1508 and 22 hits, respectively. The codominant hits reflected 12 loci and ten of these were in fact QTLs. Since the early history of genetics there has been much debate on the genetic and physiological basis for dominance (Lynch & Walsh 1998). Kacser & Burns (1981) provided an elegant molecular explanation for dominance based on the flux in a biochemical pathway composed of many interacting enzymes. They showed that dominance is expected as a reduction to 50% activity of an individual enzyme in a loss-of-function heterozygote will often have a negligible effect on the total flux in the system and thus on the phenotype. Dominance may also occur because of dominant negative mutations, inactivating a certain biochemical function, or gain-of-function mutations, such as a mutation that leads to constitutive activation of a strictly regulated molecule. So, for which type of genes and mutations is the heterozygote expected to give an intermediate phenotype? The Kacser & Burns theory also predicts that alleles with small differences in enzyme activity are likely to give intermediate heterozygotes because of the hyperbolic relationship between enzyme activity and flux. Genes encoding molecules that are rate-limiting in a biochemical pathway are also expected to be associated with intermediate heterozygotes. Thus the molecular characterization of some of the major codominant QTLs detected in this study is of major general interest.

The rather low early growth (up to 46 days of age) of the F_2 chickens was unexpected and has no obvious explanation. It could have a biological basis and represent a mild form of hybrid dysgenesis. It is well known that a reduced fitness may be observed in the F_2 generation of wide crosses and it has been attributed to possible epistatic interactions (Falconer 1981). Interestingly, this possible explanation is in fact supported by our study of epistasis in the same cross as we observed a considerable amount of epistasis for early growth but not for late growth (Carlborg *et al.* 2003). Another possibility is that the single outbred, red junglefowl founder male was not representative of the red junglefowl population as regards early growth. Furthermore, we cannot exclude that the low early growth was caused by an unknown environmental factor as the growth of the F_2 and parental populations were measured under the same environmental conditions but not in the same time period for practical reasons.

There is some overlap between the QTL positions detected in the present study and those detected in previous studies. Van Kaam *et al.* (1999a, b) performed a genome scan for growth and carcass composition using a cross between two broiler lines. Only one QTL reached genome-wise significance. This was a growth QTL located at chromosome 1 at 235 cM (Van Kaam *et al.* 1999a), thus far away from the two growth QTLs detected at chromosome 1 in the present study. However, a suggestive QTL affecting carcass percentage was detected in the vicinity of our *Growth2* QTL on chromosome 1 (Van Kaam *et al.* 1999b). Tatsuda & Fujinaka (2001a, b) identified three highly significant QTLs affecting body weight or fat deposition using an intercross between a Japanese native breed (Satsumadori) and White Plymouth Rock broilers but none of these overlapped with the QTL regions identified in our study. There is more overlap between the results of our QTL study and a recently published QTL study involving an intercross between a White Leghorn line and a commercial broiler sire line (Sewalem *et al.* 2002). Our *Growth1* on chromosome 1 maps to approximately the same region as a suggestive QTL for body weight at 9 weeks in the Leghorn × broiler intercross. However, the small effect excludes the possibility that this locus reflects the segregation of the same alleles at *Growth1* as detected in this study. Furthermore, our *Growth2, 7, 9, 10* and *13* on chromosomes 1, 4, 7, 8 and 27, respectively, maps to approximately the same region as QTLs for body weight at 9 weeks in the Broiler intercross. However, the poor precision in map positions in both studies excludes any firm conclusions about the possible identity of segregating QTLs in the two studies.

The major QTL for growth located around position 68 cM on chromosome 1 explains a large proportion of the difference in adult body size as well as in the size of eggs between the two founder populations in this study. In our previous study we observed that this chromosomal region also shows a highly significant effect on one behavioural trait, tonic immobility considered as a measure of the fear response (Schütz *et al.* 2002); the White Leghorn allele (associated with faster growth and larger eggs) was associated with a longer period of tonic immobility. Future studies will show whether the colocalization of QTLs for growth and

behaviour is a coincidence or because of a single pleiotropic QTL. It is obvious that *Growth1* must have been one of the major loci responding to selection for growth and/or improved egg production in the domestic chicken. It is an open question whether the favourable QTL allele was selected in modern time (during the 20th century) or early during the domestication of chickens. The fact that Sewalem *et al.* (2002) did not observe the segregation of a major QTL in this region in their Leghorn/broiler intercross suggests that the divergence of the *Growth1* alleles predates the development of specialized layer and broiler lines during the last century. A molecular characterization of this QTL will make it possible to trace its evolutionary history.

For most QTLs reported in this study any obvious positional candidate genes using the current, rather sparse, chicken genetic map (Schmid *et al.* 2000) were not identified. However, *Growth13* maps to the same region on chromosome 27 as the growth hormone (*GH*) gene and *Growth14* maps to the same region on the Z chromosome as the growth hormone receptor (*GHR*) gene and the prolactin receptor (*PRLR*). It has been previously reported that mutations in *GHR* cause sex-linked dwarfism in the chicken (Burnside *et al.* 1991) and *GHR* is thus an interesting positional candidate gene for this growth QTL.

Haldane's (1926) prediction of a higher recombination rate in the homogametic sex is supported by empirical data in various species. Accordingly, there is a general trend towards a higher female recombination rate in mammals. In pigs there is on average 40% excess of female recombination (Marklund *et al.* 1996; Bidanel *et al.* 2001) and the corresponding female excess in humans is about 70% (Morton 1991). Chicken appears to be an exception to this rule and shows no clear overall trend as regards sex differences. We observed an 8% higher recombination rate in the heterogametic sex (females) whereas Groenen *et al.* (1998) reported a very weak trend (+1%) in the opposite direction. This study shows that there exist highly significant sex differences in the recombination rate in certain chromosome regions but the direction varies from region to region.

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III

Many QTLs with small additive effects are associated with a large difference in growth between two selection lines in chickens

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ABSTRACT

Two growth selected lines in chickens have been developed from a single founder population by divergent selection for body weight at 56 days of age. After more than 40 generations of selection they show a nine-fold difference in body weight at selection age and large differences in growth rate, appetite, fat deposition, and metabolic characteristics. We have generated a large intercross between these lines comprising more than 800 F_2 birds. QTL mapping revealed 13 loci affecting growth. The most striking observation was that the allele in the high weight line in all cases was associated with enhanced growth, but each locus explained only a small portion of the phenotypic variance using a standard QTL model (1.3 to 3.1%). This result is in sharp contrast to our previous study where we report that the two-fold difference in adult body size between the red junglefowl and White Leghorn domestic chickens is explained by a small number of QTLs with large additive effects. Furthermore, no QTLs for anorexia or antibody response were detected despite large differences for these traits between the founder lines. The result is an excellent example where a large phenotypic difference between populations occurs in the apparent absence of any single locus with large phenotypic effects. The study underscores the need for powerful experimental designs in genetic studies of multifactorial traits. No QTL at all would have reached genome-wide significance

using a less powerful design (e.g. ~200 F_2 individuals) regardless of the huge phenotypic difference between the founder lines.

INTRODUCTION

A number of selection experiments have revealed that remarkable selection responses can be obtained for almost any multifactorial trait in plants and animals (FALCONER and MACKAY 1996; LYNCH and WALSH 1998). An excellent example of this genetic plasticity is two selection lines in chickens that have been established by divergent selection on a single trait, body weight at 56 days of age (DUNNINGTON and SIEGEL 1996). This selection experiment was initiated 1957 by crossing seven partially inbred lines of White Plymouth Rock chickens. After more than 40 generations of selection in opposite directions, the high and low weight lines show a remarkable nine-fold difference in 56 day body weight (Fig. 1).

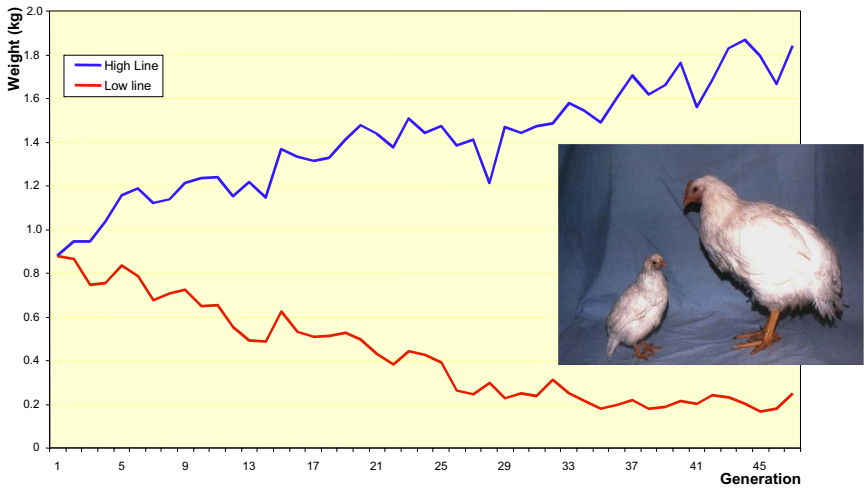

Figure 1 Body weight at 56 days of age from generation 1 to 47 of males from the chicken lines selected for high and low body weight. The chickens in the photo are from generation 37 and are 56 days old.

A number of interesting correlated responses have been observed between the two lines, including large differences in appetite. High line chickens are hyperphagic whereas low line chickens have very low appetite and tend to be anorectic (BURKHART *et al.* 1983). As a consequence, the high line chickens are feed restricted after 56 days of age (selection age) to avoid severe metabolic disorders. In contrast, anorexic individuals are observed in the low line. It became evident around generation 25 that a considerable number of females did not enter egg production and this was having an effect on selection

intensity (SIEGEL and DUNNINGTON 1987). In recent generations 5 to 20% of the chicks fail to survive during the first couple of weeks after hatch because they simply never start to eat and a proportion of the females fails to reach sexual maturity (commence egg production). These individuals can, however, be brought into egg production when force fed (ZELENKA *et al.* 1988). This anorexic condition is similar to that reported in humans (FRISCH 2002). Furthermore, the high line tends to develop obesity whereas the low line is extremely lean.

A less expected correlated response is that antibody response is greater in the low than high line following immunization with sheep red blood cells (SRBC). Interestingly, two independent studies on divergent selection for SRBC antibody response have revealed a corresponding correlated response so that a higher body weight was obtained in the lines selected for low antibody response (BOA-AMPONSEM *et al.* 1998; PARAMENTIER *et al.* 1996; PINARD VAN DER LAAN *et al.* 1998). Furthermore, a comparison of the immune response of a 2001 commercial broiler line with a 1957 randombred control line also revealed a negative correlation between growth and antibody production (CHEEMA *et al.* 2003). Thus, competition for resources between growth and immunocompetence may cause these correlations.

We have generated a large intercross population between the high and low lines as a resource for genetic dissection of QTLs that have responded to the divergent selection. The size of the experiment (>800 F_2 animals in large half-sib families) was chosen to allow the detection of QTLs with small and moderate effects. A genome scan based on 145 genetic markers covering about 80% of the chicken genome is reported here.

MATERIALS AND METHODS

Animals

The two selection lines which formed the parental population for the experiment were developed and maintained at the Virginia Polytechnic Institute and State University in Blacksburg, Virginia, USA. The common founder population originated from crosses of seven partially inbred lines of White Plymouth Rock chickens. The two lines have been maintained as closed populations selected for either high or low body weight at 56 days of age (LIU *et al.* 1994; DUNNINGTON and SIEGEL 1996). The only conscious husbandry modification made through time was that vaccination for Marek's disease was commenced in generation 18. From generation 41 of this long-term selection experiment, a reciprocal intercross was designed so that 10 high weight males were mated to 22 low weight females

and 8 low weight males were mated to 19 high weight females. From the F_1 generation, 8 males and 75 females were intercrossed and 874 F_2 chickens from a single hatch were used for the QTL study. All phenotype recordings were performed on males and females in the facilities where the selection experiment was conducted using the same dietary formulation of a corn soybean mash ration containing 20% crude protein and 2,685 kcal ME/kg of diet. Feed and water were provided *ad libitum*. Rearing was on wood shavings in 16 floor pens of about 50 chickens each in the same windowless house as where the lines underwent selection. Lighting was continuous to day 28 after which the photoperiod was from 0200 to 2200 hours. Blood samples for DNA preparations were collected at 35 days of age and a second sample was collected at 70 days of age in those cases where the amount of blood obtained at 35 days were too limited.

Phenotypic Traits

The body weight of each F_2 chicken was obtained at hatch, 14, 28, 42, 56 and 70 days of age. Packed cell volume (PCV) was measured at 39 days of age using standard methods with microhemocrit capillary tubes. Blood protein was measured at 49 days with the Veterinary Refractometer A300CO (Altago Ltd., Tokyo, Japan). At 49 days of age chickens received an injection into the brachial vein of 0.1 ml of a 0.5% suspension of sheep red blood cells (SRBC) antigen (SIEGEL AND GROSS 1980; MARTIN *et al.* 1990). Five days later a sample of 0.5 ml of blood was obtained from the brachial vein of each individual and transferred to tubes containing two drops of 5.5% EDTA. Blood samples were stored at 8°C overnight to allow the blood cells and plasma to separate. Antibody determinations were made following the microtiter hemagglutination procedures of WEGMANN and SMITHIES (1966). Titers are expressed as the $\log_2$ of the reciprocal of the last dilution in which agglutination was microscopically observed.

A number of F_2 birds died because of anorexia as we previously observed among the low line birds (DUNNINGTON and SIEGEL 1996). These F_2 individuals died early posthatch because either they did not start to eat after hatch or their feed intake was inadequate for survival. We used two classifications related to the anorexia phenotype; death, defined as 2 if the bird lived throughout the experiment and 1 if they died, and survival, where the birds were assigned the number of weeks they survived.

Linkage Map

A genetic map comprising 26 linkage groups was established based on 145 genetic markers (JACOBSSON *et al.* 2004). The total map length, summarizing the intervals flanked by markers, was 2521.9 cM. The average distance between adjacent markers assigned to linkage groups was 17.0 cM. However, there were seven gaps greater than 40 cM. The average information content at marker positions was 0.72 when information on flanking markers was taken into account. With few exceptions, the derived linkage map was in excellent agreement with the chicken consensus map (SCHMID *et al*. 2000) and with the chicken genome assembly (INTERNATIONAL CHICKEN GENOME SEQUENCING CONSORTIUM 2004). We estimated that our linkage map covers about 3180 cM corresponding to ~80% of the chicken genome (total map distance is ~4000 cM). This estimate was obtained by adding 20 cM on each side of each linkage group and by counting each single marker, showing no linkage to other markers, as covering 40 cM (20 cM on each side). The estimated map distance exceeding 40 cM for gaps larger than 40 cM was subtracted from the total map length. We assumed that any major QTL located within 20 cM of a single marker should result in at least suggestive evidence for linkage given the large F_2 material. This leaves ~20% of the chicken genome, including 13 microchromosomes, that was not covered in the present genome scan.

Statistical Analysis

An analysis of variance (ANOVA) was performed using the Minitab software (Minitab 2000) to identify sources affecting phenotypic variation. Effects of sex and/or family were significant and were therefore included as fixed effects in the model for QTL analysis. Residuals derived from the ANOVA were used as dependent variable in the regression analysis for QTL mapping. Fixed effects used in the QTL analysis of each trait are listed in Table 1.

Programs based on the least squares method for outbred populations were employed for QTL analysis of the autosomes (HALEY *et al.* 1994). Marker genotypes were used to estimate probabilities of the parental-origin of each gamete at 1 cM intervals through the genome. These conditional probabilities given marker genotypes were used to calculate coefficients of additive and dominance components for a putative QTL at each position under the assumption that the QTL was fixed for alternative alleles in the high and low parental line. The phenotypic data were regressed onto these coefficients in intervals of 1 cM. At each position, an F test for QTL segregation was carried out. The Web-based QTL Express program was used for this single QTL analysis (http://qtl.cap.ed.ac.uk; SEATON *et al*. 2000).

Table 1. Summary of the studied phenotypes with fixed effects included in the QTL analyses

Trait	n[1]	Mean	SD	Fixed effects
Body weight at hatch (g)	874	27.8	2.1	Family
at 14 days (g)	874	75.2	14.9	Family, sex
at 28 days (g)	871	179.1	56.8	Family, sex
at 42 days (g)	809	365.5	113.1	Family, sex
at 56 days (g)	795	621.6	186.9	Family, sex
at 70 days (g)	789	943.3	262.1	Family, sex
Response to SRBC (titer)	798	6.7	3.4	Family
Packed Cell Volume (% cells)	715	33.8	4.1	Family
Blood protein (g/100ml)	800	39.3	3.5	Family
Growth 0 - 14 days (g)	874	47.4	14.7	Family, sex
14 - 28 days (g)	871	103.8	47.3	Family, sex
28 - 42 days (g)	809	179.5	68.1	Family, sex
42 - 56 days (g)	794	251.7	88.6	Family, sex
56 - 70 days (g)	788	320.7	94.9	Sex

[1] number of individuals

The additive and dominance regression indicator variables for the most significant QTLs detected in the initial scan were added as covariates and a new genome scan was done using the updated model. Inclusion of the previously detected QTLs to the model should decrease the residual error variance and thereby increase the statistical power to detect QTLs with smaller effects (JANSEN 1993; ZENG 1993). Coefficients of additive and dominance components for putative QTL at each position through the genome computed by QTL Express were transferred to the QTL Fast program (CARLBORG and ANDERSSON 2002; LJUNGBERG *et al.* 2003) for these analyses. QTL analysis for the Z chromosome was performed using Qxpak based on the dosage compensation model (PÉREZ-ENCISO and MISZTAL 2004).

Genome-wide and chromosome-specific empirical significance levels of the test statistic were established by randomization using 1,000 permutations of data (CHURCHILL and DOERGE 1994). Genome-wide thresholds for highly significant (α=0.01) and significant linkage (α=0.05) were employed as proposed by LANDER and KRUGLYAK (1995). Since there is significant length heterogeneity among chicken chromosomes, thresholds for chromosome-wide significance varied considerably among chromosomes depending on the number of markers and the map length. Therefore, the chromosome-wide 5% significance levels for chromosome 4 were used as a suggestive significance threshold for each trait. The value for chromosome 4 was chosen because the map length of this chromosome constitutes

approximately 5% of the total chicken genetic map length (i.e. about 4000 cM). Thus, by using this suggestive significance threshold we expected to observe one type I error on average per genome scan and trait. Regression analysis to estimate the residual variance explained by the detected QTLs was conducted using Minitab (Minitab 2000).

RESULTS

QTL Analysis of Growth

Descriptive statistics for the phenotypic traits analyzed in this study are compiled in Table 1. No QTL for weight at hatch was found which was not unexpected since it is long known that this trait primarily reflects the phenotype of the dam rather than the genotype of the progeny (HALBERSLEBEN and MUSSEHL 1922). The results of the QTL analysis of growth and body weight traits are summarized in Table 2. Our interpretation of these data is that they reflect 13 different loci, denoted *Growth1* to *Growth13*. The presence of more than one QTL on some chromosomes was investigated by examining the QTL graphs for each chromosome. However, a second QTL was only inferred in those cases where the statistical significance was maintained even when the primary QTL (the one with the strongest statistical support) on the same chromosome was included as a cofactor in the QTL analysis. The allele derived from the high weight line was associated with enhanced growth for all loci. This suggests that the majority of these loci are true QTLs, although only five reached genome-wide significance. With the exception of *Growth11* and *13*, all loci showed largely additive effects (Table 2). The two suggestive QTLs, *Growth11* and *13*, showed negative overdominance implying a reduced growth in the heterozygotes.

The strong bias for QTL alleles inherited from the high line to be associated with high growth is illustrated in Fig. 2 where the estimated additive (*a*) substitution effect is plotted across the genome. A positive *a* value, implying enhanced growth associated with the allele from the high line, was observed on 22 out of 25 autosomes and for 77% of the genome. The data clearly illustrate that many loci across the genome have responded to the selection. None of the peaks showing a negative *a* value, implying high growth associated with the low line allele, reached even suggestive significance.

Each QTL explained only a small portion of the phenotypic variance, 1.3 to 3.1%, in the F2 generation (Table 2). We included all QTLs except Growth11 and 13, which did not show any significant additive effect, in a joint least squares analysis to estimate their individual effect as well as their combined effect on body weight at 56 days (Table 3). Since most of these QTLs appear to represent

Table 2. Quantitative Trait Loci (QTL) for body weight (BW) and growth (GR) detected in an intercross between two chicken lines divergently selected for growth to 56 days of ages. Body weights were obtained at hatch, and at 14, 28, 42, 56, and 70 days of age. Growth between body weight measurements was also calculated. Test statistics, estimated QTL effects, and the percentage of residual variance explained by each QTL are given. The QTLs are numbered *Growth1* (G1) to *Growth13* (G13).

QTL	Chr	Pos. cM	Trait (g)	F[1]	Additive effect ± SE[2]	Dominance effect ± SE[2]	Var (%)[3]	Marker1[4]	Marker2[4]
G1	1	437	GR56-70	11.3**	19.7±4.2	-5.2±6.4	2.8	*LEI062*	*LEI134*
G2	2	115	BW56	6.1†	26.8±7.9	12.8±11.9	1.5	*MCW239*	*MCW293*
G3	2	253	BW70	6.2†	37.5±11.2	-17.2±17.5	1.6	*LEI147*	*MCW245*
G4	3	123	BW28	5.7†	10.6±3.2	-6.5±6.1	1.3	*ADL155*	*ADL371*
G5	3	243	BW42	6.2†	21.0±6.0	-7.5±10.7	1.5	*MCW207*	*LEI065*
		252	BW56	5.8†	35.4±10.4	4.3±20.0	1.4		
G6	4	50	GR42-56	6.4†	23.0±6.6	-9.5±17.5	1.6	*ADL317*	*ADL145*
		51	BW56	7.8†	54.2±14.0	-21.0±37.1	1.9		
		52	GR56-70	9.7**	30.3±6.9	8.5±18.1	2.4		
		54	BW70	9.0*	77.8±18.4	-8.6±47.9	2.2		
		62	GR28-42	7.2†	18.2±4.8	4.7±11.3	1.8		
		62	BW42	6.4†	28.6±8.0	4.2±18.7	1.6		
G7	4	148	GR28-42	6.3†	13.2±3.8	4.6±6.9	1.5	*LEI122*	*LEI076*
		149	BW42	7.4†	23.9±6.3	9.8±11.7	1.8		
		151	BW70	8.3*	56.3±14.0	16.6±26.8	2.1		
		151	BW56	6.8†	37.4±10.5	20.1±20.1	1.7		
		151	BW28	6.1†	11.3±3.3	4.6±6.3	1.4		
G8	5	107	BW70	7.0†	42.4±11.8	25.3±18.6	1.8	*MCW038*	*MCW029*
G9	7	42	GR42-56	9.3*	16.7±3.9	-4.0±6.1	2.3	*MCW236*	*MCW120*
		43	BW56	12.6**	41.0±8.4	-15.0±13.0	3.1		
		44	BW42	9.3*	21.7±5.2	-9.0±8.1	2.3		
		44	GR28-42	8.5*	12.3±3.2	-6.6±4.9	2.1		
		63	GR56-70	6.5†	17.8±5.0	4.9±9.1	1.6		
		66	BW70	10.3**	63.6±14.0	-13.4±26.6	2.6		
G10	13	0	BW42	5.6†	16.0±5.5	13.8±8.8	1.4	*ADL147*	*MCW213*
		4	BW70	6.5†	35.0±11.1	27.2±16.4	1.6		
		4	GR56-70	5.9†	13.6±4.1	5.2±6.1	1.5		
G11	20	9	BW70	6.1†	17.8±16.2	–110.5±34.8	1.5	*ADL125*	*HCK*
G12	20	61	GR0-14	13.4**	4.6±0.9	-2.1±1.9	3.1	*HCK*	*MC3R*
		62	BW14	12.7**	4.5±0.9	-2.1±1.9	2.9		
G13	28	0	BW14	6.0†	1.3±0.8	-4.4±1.4	1.4	*MCW227*	*MCW227*

[1] F statistic for the QTL and level of significance; ** Genome-wide 1% significance, * Genome-wide 5% significance, and † Suggestive 5% significance.

[2] The additive and the dominance effects were defined as the deviation of animals homozygous for the high line allele or heterozygous, respectively, from the mean of the two homozygotes. SE=standard error.

[3] Reduction of residual variance for the F_2 population when including a QTL at the given position.

[4] Markers flanking the QTL interval estimated by the one-LOD drop method.

true QTLs it was of interest to estimate their effect on body weight at 56 days, the sole criteria for selection when developing the two body weight lines. The results indicate that these 11 loci explain at most 50% of the phenotypic difference between the founder lines and ~13% of the residual variance in the F_2 generation. This is most likely an overestimation because some QTLs could be false positives and some estimated QTL effects maybe inflated (see Discussion).

Table 3. The body weight in the parental lines and the estimated additive (a) effects on body weight at 56 days for 11 Quantitative Trait Loci (QTLs) identified in the high (H) x Low (L) intercross

H X L cross, body weight at 56 days of age (in grams)		
Parentals		
H line	1522	
L line	181	
H – L difference	1341	
F_2 generation		
QTL[1]	a ±SE	$2a^2$
Growth1	24.5±12.2	49.0
Growth2	22.4±7.5	44.8
Growth3	27.9±8.3	55.8
Growth4	27.8±9.5	55.6
Growth5	37.4±9.8	74.8
Growth6	46.0±13.4	92.0
Growth7	33.1±10.0	66.2
Growth8	21.9±8.6	43.8
Growth9	39.3±8.0	78.6
Growth10	21.5±7.9	43.0
Growth12	15.2±7.8	30.4
Sum		634.0
% population difference		47.3
% residual variance		13.3

[1]*Growth11* and *Growth13* showed no significant additive effects and were therefore not included in this analysis.
[2]The additive effect represents by definition half the estimated phenotypic difference between the two homozygotes. Therefore we provide the estimates for 2a here.

QTL Analysis of Anorexia

Anorexia occurs regularly in our low line but it has not been observed in our high line or in F_1 crosses of the lines. Therefore it was surprising that as many as 18% of the F_2 birds died before 56 days of age. We assume that a large proportion of these birds died due to

anorexia because there was no evidence of infectious diseases and the veterinary record stated that the chickens were of excellent health. We were only able to obtain blood samples for DNA preparation from 60 of the 176 birds that died. The QTL analysis of this trait did not reveal any significant locus, not even at the suggestive level. We then asked whether any of the 13 growth QTLs had a significant effect on the incidence of anorexia. In this case we could use nominal significance thresholds because we did not conduct a genome-wide search, but no QTL showed a significant effect. However, there was a weak trend that QTL alleles from the high line were associated with higher survival, the estimated additive effect for survival showed a small, but positive value for 11 out of 13 growth QTLs.

No evidence for segregation distortion

If a major susceptibility locus was underlying the high incidence of anorexia in the F_2 generation we expected to observe segregation distortion at that locus because we were unable to sample 118 out of the 176 F_2 birds that died before 10 weeks of age. We therefore carried an analysis of segregation distortion in this material using the QTL express program. We observed in total seven regions that showed a significant deviation at the nominal significance thresholds ($P<0.05$) either for the additive component (deviation from 1:1 segregation) or the dominance component (deviation from 50% heterozygotes). This is not more than expected by chance given the large number of tests carried out here; a test was carried out at each cM across our linkage map based on 145 markers). Thus, there was no global evidence for segregation distortion.

We then asked the question if there were any signs of segregation distortion at the position for the growth QTLs. No strong deviations were observed, however there was a trend towards an excess of alleles from the high line at QTLs with 10 out of 13 positions being positive (Table 4).

One of these deviations was significant (*Growth8* on chromosome 5) and another one approached significance (*Growth2* on chromosome 2). This result is consistent with the QTL analysis of anorexia showing that although none of the growth QTLs had a major impact on the incidence of anorexia, they may contribute each with a small effect.

Table 4. Analysis of segregation distortion at QTL positions in the F_2 generation of the high x low intercross

		Additive component		Dominance component	
QTL	Chr:Pos	a	t	d	t
Growth1	1:437	0.003	0.15	0.509	0.63
Growth2	2:115	0.043	1.84(*)	0.484	-1.07
Growth3	2:253	0.016	0.70	0.498	-0.16
Growth4	3:123	0.004	0.27	0.497	-0.40
Growth5	3:252	-0.004	-0.21	0.495	-0.64
Growth6	4:51	0.005	0.38	0.501	0.15
Growth6	4:62	0.010	0.67	0.505	0.75
Growth7	4:151	0.011	0.96	0.508	0.51
Growth8	5:107	0.044	2.06*	0.512	0.81
Growth9	7:43	0.001	0.03	0.506	0.38
Growth10	13:4	-0.005	-0.56	0.493	0.51
Growth11	20:9	0.027	0.72	0.514	1.76
Growth12	20:62	0.017	1.02	0.503	0.88
Growth13	28:0	-0.020	-0.89	0.502	0.11

a= additive component, a value above 0 indicate an excess of alleles from the high line
d= dominance component, estimated frequency of High/Low heterozygotes. The expected frequency is 0.500.
t=Student's t test
*P<0.05; (*)P<0.10

QTL Analysis of Packed Cell Volume (PCV), Blood Protein and Antibody Response to Sheep Red Blood Cells (SRBC)

Metabolic needs for growth, reproduction, and immunocompetence vary among the selected lines. For gross measures of physiological demands, we measured PCV which is associated with oxygen carrying capacity and total blood protein which is associated with reserves needed for growth and for coping with environmental insults. One of the striking correlated responses that have been obtained for these two selection lines is that the high line shows a poor antibody response to immunization with SRBC (LIU *et al.* 1995); F_1 crosses show a higher response than either parental line with a heterosis of 70%. Based on this observed line difference one might expect a negative phenotypic correlation between growth and immune traits in the F_2 generation. However, the correlation analysis revealed a weak positive association between 56 day body weight and response to SRBC ($r = 0.13$, $P<0.0001$), and PCV ($r = 0.09$, $P=0.02$) as well as between the 56-day body weight and blood protein level ($r = 0.17$, $P<0.0001$). Furthermore, no significant QTL was detected for these traits and none of the 13 growth QTLs showed a significant effect on SRBC antibody response, not even at the nominal level.

DISCUSSION

This study revealed 13 significant or suggestive QTLs for growth, each explaining only a small portion of the residual phenotypic variance (1.3 to 3.1%) in the F_2 generation. We concluded that the majority of these QTLs are true QTLs, although only five reached genome-wide significance, because the allele from the high line was associated with higher growth at all 13 QTLs (Fig. 2; Table 3). This is an unlikely outcome if many of these loci are false positives. Furthermore, our conclusion is supported by the results of a recent global search for epistatic interaction that revealed nine different pairs of interactions involving seven different loci in total (Ö. CARLBORG *et al.* unpublished). As many as six of these mapped in

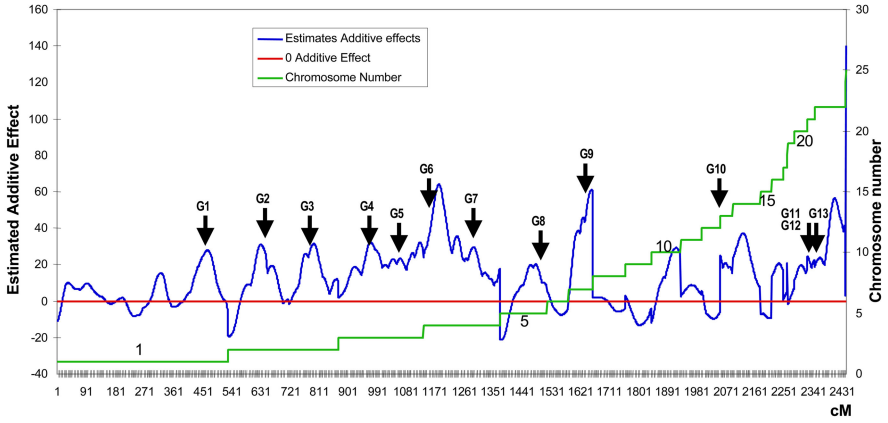

Figure 2 Plot of the estimated additive (*a*) effects across the chicken genome in a QTL analysis of body weight at 56 days based on an intercross between the high and low growth lines. A positive *a* value indicates that the allele from the high line is associated with high growth. The peak positions of the QTLs detected by segregation analysis are indicated.

the near vicinity of QTLs reported in the present study (*Growth 2, 4, 6, 9, 10* and *12*). The detected QTL explained "only" about 50% of the line difference (Table 3). However, our data on the reciprocal F_1 generations indicated that about 100 gram of the line difference is due to maternal effects (data not shown). Thus, even if all true QTLs were known they would not explain the entire line difference.

Our observation of many QTLs, each with small individual effects is consistent with the steady response to selection, without any major leaps, that has been observed during the course of the selection experiment (Figure 1). The data show that the dramatic response to selection has not involved any QTL with large individual effects, although we cannot exclude the possibility that a major QTL is hiding in the ~20% of the chicken genome that was not covered in

this study. The size of the individual QTL effects is difficult to assess after this initial genome scan for several reasons. It is likely that the some estimated QTL effects have been inflated since a common problem in QTL studies is that those loci where the effect by chance is overestimated are more likely to reach statistical significance (MCKINNON and GEORGES 1992; GORING *et al.* 2001). It is also possible that individual QTL effects have been overestimated because they represent a haplotype effect of two or more linked QTLs, each with a smaller individual effect. Furthermore, it is possible that some QTL effects have been underestimated if QTLs were not fixed for different alleles in the founder lines. The statistical analysis has been carried out with the assumption that the lines are fixed for different alleles, but if this assumption is not fulfilled the effects are underestimated. Another possible bias when estimating QTL effects maybe caused by the fact that a sizable portion of the birds died because of anorexia and this may also diminish the estimated effects. However, the segregation analysis indicated that no or only a very minor segregation distortion occurred at QTLs so this possible bias should not seriously affect the estimates. The importance of the QTLs may also have been underestimated due to epistatic interaction since only the marginal effects of the individual loci are revealed in a standard one-dimensional QTL search (CARLBORG *et al.* 2003). Finally, the rather small QTL effects, estimated as the percentage of the residual variance explained by each QTL, are partly due to the very large variance observed in the F_2 generation. For instance, the additive effect of the *Growth9* QTL on chromosome 7, 41 grams, maybe compared with the estimated standard deviations of 33 and 212 grams for the low and high founder lines, respectively.

Our finding of many QTLs, each with small individual effects is in good agreement with the results of previous QTL studies of intercrosses between mouse lines, divergently selected for growth, and corn lines, divergently selected for oil content in kernels (CHEVERUD *et al.* 1996; MORRIS *et al.* 1999; LAURIE *et al.* 2004). Similarly, VAN KAAM *et al.* (1999) detected only a few QTLs each with a small effect using an intercross between broiler lines selected for high growth, despite a powerful experimental design involving progeny testing. The results, however, are in sharp contrast to our previous QTL study based on an intercross between red junglefowl and White Leghorn chickens where we documented that a few QTLs with large effects explain a large portion (~70%) of the difference between the founder lines in adult body weight and a large part (~30%) of the residual phenotypic variance in the F_2 generation (KERJE *et al.* 2003). The experimental design, regarding the size of the pedigree and the number of genetic markers, of the two studies are very similar. However, the characteristics of the founder populations are strikingly different. The red junglefowl and White Leghorn

chickens have been separated for thousands of years whereas the high and low lines were developed from a common ancestral population during 41 generations of intensive selection for the single trait 56 day body weight. The former show a two-fold difference in adult body weight whereas the latter show a nine-fold difference in body weight at 56 days of age. The number of QTLs detected in the two studies is similar, but the distribution of effects is very different. There has been no intensive selection for body weight in White Leghorns in recent years. The QTLs with major effects on body weight may have been fixed before advanced forms of animal breeding were implemented. Our results suggest that no QTL with a large individual effect on growth was segregating in the founder population for the high and low lines. Despite this, a remarkable selection response has been obtained which illustrates the genetic plasticity of most biological traits provided that sufficient genetic diversity exists in the population under selection. In this context it is of interest that a very high nucleotide diversity of about five single nucleotide polymorphisms (SNPs) per kilobase has been documented in comparisons both between and within breeds of domestic chicken (INTERNATIONAL CHICKEN POLYMORPHISM MAP CONSORTIUM 2004). This is about five-fold higher than the nucleotide diversity occurring in humans across populations (INTERNATIONAL SNP MAP WORKING GROUP 2001). Thus, there must be many variants with minor effects on gene expression or gene function that can contribute to a selection response like the one observed for our high and low lines. Thus, the distribution of observed QTL effects in a QTL mapping experiment will depend on the genetic background of the population(s) investigated and a huge phenotypic difference between two populations does not necessarily imply the existence of QTLs with large effects.

Our high and low body weight lines provide interesting models for metabolic disorders in humans. The low line shows a high incidence of anorexia and is very lean. In contrast the high line shows hyperphagia, obesity, and impaired glucose tolerance not associated with insulin deficiency (DUNNINGTON and SIEGEL 1996), the latter a classical feature of Type II diabetes in humans. Furthermore, electrolytic lesion of the ventro-medial hypothalamus has shown that birds from the high line have a defect in the hypothalamic satiety mechanism (BURKHART *et al.* 1983). The great majority of clinical cases of metabolic disorders in humans have a polygenic background and the present study shows that such disorders may have a strong genetic background even in the absence of mutations with major effects. A very large human dataset would be required to detect loci explaining as little as a few percent of the phenotypic variance for a disorder. An important question for the usefulness of our chicken intercross as a model for metabolic disorders in humans is whether it is possible to identify the mutations underlying these QTLs despite

their minor effects. This should be possible unless the majority of the QTLs are due to the combined effect of several closely linked mutations each with a minute effect. We are maintaining an advanced intercross line (AIL; DARVASI AND SOLLER 1995) for high-resolution mapping that are now (year 2005) at the F_8 generation. High-resolution mapping in the chicken is facilitated by the high recombination rate ranging from 2.5 to 21 cM/Mbp depending on chromosome (INTERNATIONAL CHICKEN GENOME SEQUENCING CONSORTIUM 2004). Here we have analyzed each trait separately, but it is known that multivariate (multitrait) techniques help to the resolution of QTL (TURRI *et al.* 2004). The wide collection of correlated traits recorded in this experiment should also allow us to benefit from multitrait analyses. Positional cloning of QTLs in chicken is now greatly facilitated by the access to a high quality draft genome sequence (INTERNATIONAL CHICKEN GENOME SEQUENCING CONSORTIUM 2004) and a SNP map comprising 2.8 million loci (INTERNATIONAL CHICKEN POLYMORPHISM MAP CONSORTIUM 2004).

We did not observe any QTLs for anorexia, despite a large difference in incidence between the two lines (DUNNINGTON and SIEGEL 1996) and a high incidence in the F_2 generation. We propose that this condition is caused by a threshold effect rather than a few predisposing loci. This means that the combined effect of many QTL alleles reducing appetite at one point makes the feed intake inadequate for survival. The high incidence in the low line combined with the absence of anorexia in the F_1 generation (SIEGEL and DUNNINGTON 1987; DUNNINGTON and SIEGEL 1996) suggested that a few recessive loci with major effects may underlie the incidence of anorexia in this pedigree. However, the incidence in the F_2 generation appears to be too high (almost as high as in the low line) to be consistent with a simple recessive model. This is because the allele frequency among the F_2 birds of an allele present in the low line, but absent in the high line, should be half the frequency in the low line and the phenotype frequency should thus be one fourth. Epistatic interaction in the form of unfavorable combinations of alleles/haplotypes selected in the two lines may also contribute to the high incidence of anorexia among the F_2 birds. We may also have failed to detect any QTL for anorexia partly because of (*i.*) the weak power of QTL analysis of all-or-none traits, (*ii.*) the fact that we were only able to collect DNA samples from a fraction of the birds that died, and (*iii.*) some birds died for other reasons than anorexia.

We did not detect any QTL for antibody response to SRBC, packed cell volume or total blood protein. There was a weak but significant correlation between body weight and antibody response. Furthermore, our observation that the QTLs for growth showed no significant effect on antibody response may suggest that there is no direct causal relationship between growth and antibody response. This appears unlikely because there are also two independent

experiments where selection for low immune response led to a correlated increase in body weight (BOA-AMPONSEM *et al.* 1998; PARAMENTIER *et al.* 1996; PINARD VAN DER LAAN *et al.* 1998**).** However, no QTL showing significant effects on both growth and antibody response has yet been revealed (SIWECK *et al.* 2004; this study). This suggests that the association may only be observed when the birds have passed a certain weight threshold where the conflict of resource allocation devoted to growth and the immune system becomes severe. Thus, according to this model, too few birds in the F_2 generation showed a sufficiently high growth rate to cause a general correlation between body weight and immune response. This may also explain why we did not detect any significant QTLs for antibody response.

Several previous studies have reported growth QTLs in chickens (https://acedb.asg.wur.nl/). There is some overlap between QTLs found in this study and in those previous studies but the data should be interpreted with caution due to the poor precision in initial QTL mapping experiments. It is therefore not possible to judge whether two overlapping QTLs detected in different studies represent the same locus. However, a QTL at ~400 cM on GGA1 and QTLs on GGA4 and 7 detected by KERJE *et al*. (2003) in a red junglefowl/White Leghorn intercross maps approximately to the same region as QTLs in our study. SEWALEM *et al.* (2002) made a QTL study in an intercross between layer and broiler lines. The location of one of our major QTLs, *Growth9* on GGA7, overlaps with a QTL for 21, 42 and 63 day body weight in that intercross. Also our *Growth1* and *Growth13* overlap with QTLs identified in that intercross. DEEB and LAMONT (2003) found a significant effect on 56 day body weight in Fayoumi chickens to a marker on chromosome 28 like we did, however with only one marker on chromosome 28 we cannot judge whether these two QTLs overlap or not.

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IV

QTL analysis of body composition and metabolic traits in an intercross between chicken lines divergently selected for growth

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Keywords: quantitative trait locus; correlated selection response; body composition; metabolic traits; chicken

ABSTRACT

The high and low growth lines of chickens have been developed from a single founder population by divergent selection for body weight at 56 days of age for more than 40 generations. The two lines show a nine-fold difference in body weight at selection age and several interesting correlated selection responses such as altered body composition and metabolic differences. We have generated a reciprocal intercross comprising more than 800 F_2 birds. In a previous study we reported the detection of 13 Quantitative Trait Loci (QTLs) affecting growth. Here we report QTLs for body composition (fat deposition, muscle development), weight of internal organs, and metabolic traits (plasma concentrations of glucose, insulin, cholesterol, glucagon, triglycerides, and IGF-I). Most of the QTLs with convincing statistical support mapped in the vicinity of growth QTLs. One of the most interesting observations was that the type of reciprocal cross had highly significant effects on body weight at hatch, and on plasma concentrations of glucose, cholesterol, insulin, and IGF-I but it had no significant effect on body weight at 56 days of age. The reciprocal cross explained between 15-35% of the phenotypic variance for weight at hatch, and for plasma concentrations of glucose and insulin. The observed pattern indicated that these effects were caused by maternal effects or by genetic differences in mitochondrial DNA.

INTRODUCTION

THE CHICKEN IS BECOMING a prime vertebrate model for the genetic dissection of complex phenotypic traits due to the release of a high quality draft genome sequence at 6.6X coverage (8) and a genetic map comprising 2.8 million single nucleotide polymorphisms (SNPs, 9). Other merits with the chicken include a fairly small genome size (1.06 Gbp) and a high recombination rate (8). There also exist a number of chicken lines that carry mutations causing a monogenic phenotype or that have been selected for different purposes (3). One example is the high (HW) and low weight (LW) lines developed at the Virginia Polytechnic Institute and State University (Blacksburg, Virginia) from a base population of the White Plymouth Rock breed (5, 16). The selection experiment was initiated at 1957 and after more than 40 generations of divergent selection solely on body weight at 56 days of age the two lines differ nine-fold in weight at this age. A number of correlated responses for body composition and metabolic traits have been obtained. The HW birds become obese and must be feed-restricted to avoid severe metabolic disorders whereas the LW birds tend to be anorectic and are very lean. The HW birds have elevated plasma concentrations of glucose, insulin, lipids, and glucagon and show impaired glucose tolerance (4, 5). Thus, these two lines are novel models for metabolic disorders in humans. We have generated an intercross between the HW and LW lines comprising more than 800 F_2 birds. In a previous study, we reported the identification of 13 Quantitative Trait Loci (QTLs) affecting growth (11). However, each of them explained only a small portion of the residual variance for body weight at 56 days in the F_2 generation (1.3 - 3.1%) and combined they explain at most ~50% of the difference between the two lines.

In this study we report the QTL analysis of body composition and metabolic traits. In addition we analyzed phenotypic differences between reciprocal crosses that may be caused by maternal effects, QTLs on sex chromosomes or genetic variation in mitochondrial DNA.

MATERIALS AND METHODS

Experimental animals

The high (HW) and low weight (LW) selection lines have been developed and maintained at the Virginia Polytechnic Institute and State University in Blacksburg, Virginia, USA (5, 16). The base population was formed by crossing seven partially inbred lines of White Plymouth Rock chickens. The selection lines have been maintained at the same location as closed populations selected for

either high or low body weight at 56 days of age. Birds representing generation 41 of this long-term selection experiment were used to generate a reciprocal F_2 intercross. Ten HW males were mated to 22 LW females and eight LW males were mated to 19 HW females to produce each reciprocal half of the cross, i.e. H x L and L x H F_1 progeny (Figure 1). From the F_1 generation, 4 HL males were intercrossed to 37 LH females and 4 LH males were intercrossed to 38 HL females. A total of 874 F_2

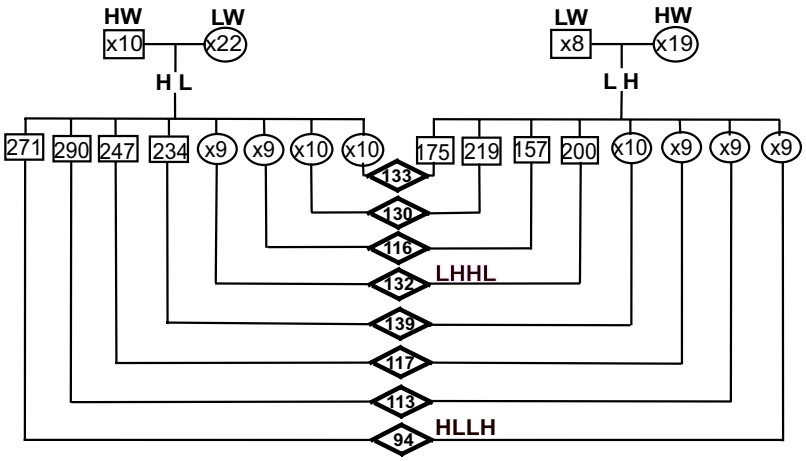

Figure 1. Pedigree structure of the F_2 intercross between two chicken lines divergently selected for growth. The F_1 sires are marked with their ID numbers. The numbers of dams mated to each sire as well as the numbers of F_2 offspring in each half-sib family are indicated.

offspring comprising 75 full-sib families were used for QTL analysis. All F_2 birds were from a single hatch. The intercross was raised using the same dietary formulation and feeding program as used for the founder lines.

Phenotype analysis

All phenotypes were recorded in the facilities where the selection experiment was conducted. The phenotypic measures included body weight at 56 days of age, and weight of abdominal fat, breast muscle, lung, shank, bursa, and spleen at 70 days of age. Mesenteric or gizzard fat were not included in the measurement of abdominal fat weight. The weight of *Pectoralis major* was collected for breast muscle weight. The weight of shank (*Metatarsus*) plus toes and lung were also recorded separately.

In addition to the body composition traits, plasma concentrations of glucose, cholesterol, triglycerides, insulin, glucagon, and IGF-I were measured. For practical reasons, it was not possible to collect

the blood samples from fasted birds although this would have been preferred. At 63 days of age, blood samples were collected via the brachial vein and plasma was separated from whole blood within one hour of collection. Plasma samples were frozen immediately and stored at –70°C. At the time of assay, samples had been thawed and refrozen for other assays one time. The concentration of glucose, cholesterol, and triglycerides were recorded using the Beckman Synchron CX system. Glucagon levels were determined using a glucagon radioimmunoassay kit (ICN Pharmaceuticals, Inc. Costa Mesa, USA). Insulin was measured by the "ImmuChem^{TM} Coated Tube Insulin ^{125}I Radioimmunoassay kit (ICN Pharmaceuticals, Inc. Costa Mesa, USA). IGF-I was also assayed by a radioimmunoassay (ALPCO Diagnostics, Windham, USA).

Several of the physiological traits showed significant deviations from normality and were transformed using the natural logarithm (i.e. glucose) or the square root (i.e. triglycerides, insulin, glucagon, and IGF-I) to remove skewness. Extreme outlying values were excluded based on an ascertainment of normality (see Table 1) in order to reduce the risk of statistical artifacts in the QTL analysis.

Table 1. Summary of phenotypic data from the F_2 generation of an intercross between the High and Low selections lines. Fixed effects and covariates included in the QTL analyses are also given.

Traits	n[a]		Mean±SD	Fixed effects	Covariate
Body weight at 56 d (g)	795	(0)	621.6±186.9	Family, sex	
Body composition at 70 d (g)					
Abdominal fat	402	(3)	5.5±4.1	Family, sex	Bw70
Breast muscle	201	(0)	91.1±28.8	Family, sex	Bw70
Lung	405	(0)	6.5±2.2	Family, sex	Bw70
Shank weight	405	(0)	42.5±12.0	Family, sex	Bw70
Bursa	405	(0)	1.9±0.7	Family	Bw70
Spleen	401	(2)	1.4±0.5		Bw70
Metabolic parameters at 63 d					
Glucose[b] (mg/dL)	782	(3)	5.5±0.1	Family	
Cholesterol (mg/dL)	785	(0)	111.3±19.7	Family, sex	
Triglycerides[c] (mg/dL)	783	(2)	7.6±1.3	Family, sex	
Insulin[c] (microIU/mL)	728	(3)	3.7±1.6	Family	
Glucagon[c] (pg/mL)	758	(6)	13.1±3.9	Family	
IGF-I[c] (ng/mL)	614	(117)	5.2±1.5	Family	

[a] Values in parentheses are the number of individuals excluded based on ascertainment of normality; [b]Data transformed using natural log; [c]Data transformed using square root. n= number of individuals; Bw70= body weight at 70 d.

Genetic marker data

Genotype data on 145 DNA markers representing 26 linkage groups have been generated for this intercross (10). The total map length, summarizing the intervals flanked by markers, was 2469.8 cM. The average distance between adjacent markers assigned to linkage groups was 17.0 cM but there were seven gaps greater than 40 cM. Average information content was 0.72 when information on flanking markers was taken into account. The map fits well with previously published linkage maps (22) and with the genome assembly of February 2004 which is available at the ENSEMBL (http://www.ensembl.org) and the UCSC (http://genome.ucsc.edu) genome browsers.

Statistical analysis

Analysis of variance (ANOVA) was performed using Minitab (18) to identify factors affecting phenotypic variation. The effects of sex and family were significant for most traits and therefore included in the model for QTL analysis (Table 1). The 70-day body weight was included as a covariate in the QTL analysis of body composition traits. Thus, all results concerning body composition traits were compared at an adjusted equal body weight. Pearson's correlation coefficients and the significance of each pair wise comparisons of traits were estimated with the correlation procedure of Minitab, and the effect of reciprocal cross (i.e. HLLH and LHHL) was analyzed using the ANOVA and regression procedures (18).

A least squares method for QTL analysis of outbred population was used for autosomes (7). Marker genotypes were used to estimate probabilities of the parental-origin of each gamete at 1 cM intervals through the genome. These conditional probabilities were used to calculate coefficients of additive and dominance components for a putative QTL at each position under the assumption that the QTL was fixed for alternative alleles in the parental lines. Residuals derived from the ANOVA were used as the dependent variable and regressed onto the additive and dominance coefficients in intervals of one cM. At each position, an F value comparing a full model with a model without a QTL was calculated. A two-QTL model was also evaluated. The web-based QTL express program (http://qtl.cap.ed.ac.uk) was used for single and two QTL analyses (23).

Inclusion of previously detected QTLs should decrease the residual variance and thereby increase the statistical power to detect QTLs with smaller effects (12, 24). Therefore, the additive and dominance regression indicator variables for the most significant single QTL in the initial analysis were added as covariates and a new genome scan was carried out using the updated model. Coefficients of additive and dominance components for the putative QTLs at each position

through the genome, computed by QTL express, were transferred to the QTL Fast program (1, 17) for these analyses. QTL mapping for the Z chromosome was performed using Qxpak based on the dosage compensation model (21).

To address the multiple testing issue in QTL scans, genome-wide and chromosome-specific empirical significance levels of the test statistic were established by randomization using 1000 permutations of data (2). Genome-wide thresholds for highly significant (α=0.01) and significant linkage (α=0.05) were employed as proposed by Lander and Kruglyak (14). The chromosome-wide 5% significance levels obtained for chromosome 4 were used as suggestive evidence for the presence of QTL because the genetic map length for this chromosome constitutes about 5% of the total map length for chicken. By using this suggestive significance threshold we expected to obtain one false positive QTL per genome scan and trait. We employed the one-LOD (logarithm of odds) drop method to estimate confidence intervals for identified QTLs at the suggestive and significant level of significance (20).

RESULTS

Descriptive statistics

Long-term artificial selection led to a divergence of approximately eight standard deviations in body weight at 56 days of age between the HW and LW lines. The high body weight in HW chickens is associated with high mean weights of abdominal fat and altered body composition. The HW line also exhibits elevated plasma levels of glucose, lipid, insulin, and IGF-I (5). The overall means and standard deviations of body weight, body composition traits, and metabolic parameters for the F_2 generation derived from a reciprocal intercross between the HW and LW lines are presented in Table 1.

A statistical analysis of the phenotypic data from the F_2 population revealed that a number of the traits were significantly correlated (Table 2). Body weight was strongly correlated with body composition traits (r=0.64 or higher). Positive correlations were also found between abdominal fat and both muscle weight and weight of internal organs (r=0.39 to 0.56). There was a weak positive correlation between abdominal fat weight and cholesterol (r=0.10; P<0.05) as well as triglycerides(r=0.20; P<0.001) content in plasma. We found weak negative correlations between the levels of insulin and glucose (r=-0.18, P<0.001), cholesterol (r=-0.13, P<0.001), and IGF-I levels (r=-0.12, P<0.01), and between the levels of glucagon and both cholesterol (r=-0.10, P<0.01) and glucose (r=-0.13, P<0.01). However, some traits did not show significant associations

suggesting that loci influencing these phenotypes may segregate independently. For example, there was no significant correlation between glucagon and insulin levels.

Table 2. Pearson's correlation coefficients among body weight, body composition, and metabolic traits in a chicken F_2 intercross population.

Trait	BW	AF	BU	SP	BM	LU	SH	CH	GL	TG	GC	IGF-I	INS
BW	1												
AF	0.66***	1											
BU	0.64***	0.39***	1										
SP	0.67***	0.46***	0.51***	1									
BM	0.95***	0.56***	0.65***	0.69**	1								
LU	0.81***	0.55***	0.58***	0.61**	0.83**	1							
SH	0.86***	0.49***	0.70***	0.64**	0.84**	0.80**	1						
CH	0.13***	0.10*	0.06	-0.02	0.00	0.04	0.10*	1					
GL	0.07	0.01	0.15**	0.06	0.14	0.04	0.15**	0.49***	1				
TG	0.17***	0.20***	0.10*	0.1*	0.20**	0.16**	0.16**	0.46***	0.22***	1			
GC	0.09*	0.08	0.07	0.07	0.09	0.18**	0.09	-0.10**	-0.13**	0.06	1		
IGF-I	0.09*	0.08	0.02	0.01	-0.03	0.01	0.00	0.05	-0.04	0.13**	0.10*	1	
INS	0.04	0.08	-0.05	0.05	0.11	0.15**	-0.02	-0.13***	-0.18***	-0.07	0.04	-0.12**	1

BW: 56-day body weight; AF: abdominal fat weight; BU: bursa weight; SP: spleen weight; BM: breast muscle weight; LU: lung weight; SH: shank weight; CH: cholesterol; GL: glucose; TG: triglycerides; GC: glucagon; IGF-I: insulin-like growth factor-I; INS: insulin. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Significant phenotypic differences between reciprocal crosses

The evaluation of possible phenotypic differences between reciprocal crosses (i.e. HLLH, LHHL) was performed for each sex separately to allow us to conclude the basis for any observed effect (Tables 3 and 4). Consistent and highly significant effects of reciprocal crossing were found in both males and females for weight at hatch and for several metabolic traits (plasma concentrations of glucose, cholesterol, insulin, and IGF-I). The effect of reciprocal crosses explained an astonishing 15-35% of the residual phenotypic variance for, weight at hatch, glucose, and insulin

concentrations. If the maternal grand-dam originated form the HW line the F_2 chickens had higher weight at hatch, higher glucose, and lower insulin concentrations (Table 3). A minor reciprocal cross effect on triglycerides concentration was only significant in females. No significant effect on 56 day body weight was found.

Table 3. Phenotypic effects of reciprocal crosses (i.e. HLLH vs. LHHL) in the F_2 generation of an intercross between two selection lines in chicken.

Trait	F_2 total		F_2 Female			F_2 Male		
	n	Means±SD	n	Effect±SE[a]	%var[b]	n	Effect±SE[a]	%var[b]
Body weight at 0 d (g)	874	27.8±2.1	438	1.9±0.2***	21.0	436	1.8±0.2***	18.4
Body weight at 56 d (g)	795	621.6±186.9	395	24.5±16.0	-	400	9.5±18.4	-
Glucose (mg/dL)	782	5.5±0.1	391	0.10±0.01***	14.4	391	0.09±0.01***	15.7
Cholesterol (mg/dL)	785	111.3±19.7	392	8.8±1.8***	5.6	393	12.1±1.9***	9.3
Triglycerides (mg/dL)	783	7.6±1.3	391	0.31±0.12*	1.6	392	0.07±0.14	-
Insulin (microIU/mL)	728	3.7±1.6	366	-2.0±0.1***	36.4	362	-1.8±0.1***	30.1
IGF-I (ng/mL)	614	5.2±1.5	310	0.72±0.17***	5.6	304	0.95±0.16***	9.9

[a]Cross substitution effect (i.e. HLLH - LHHL) estimated by regression analysis. [b]Percentage of the residual phenotypic variance explained by the reciprocal cross effect. n=number of individuals. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Table 4. Genetic constitution as regards sex chromosomes and mtDNA in a reciprocal intercross between the high and low growth selection lines in chicken.

	HL x LH		LH x HL	
	Sex chromosome	mtDNA[a]	Sex chromosome	mtDNA[a]
Mating	Z^HZ^L x Z^LW^H		Z^LZ^H x Z^HW^L	
Male F_2	Z^HZ^L, Z^LZ^L	$mtDNA^H$	Z^HZ^L, Z^HZ^H	$mtDNA^L$
Female F_2	Z^HW^H, Z^LW^H	$mtDNA^H$	Z^HW^L, Z^LW^L	$mtDNA^L$

[a]Superscript H and L indicate that the chromosome/mtDNA originated from the high or low lines, respectively.

QTL analysis of body composition and weight of internal organs

The results of the QTL analysis are summarised in Table 5 and the chromosomal location of detected QTLs are depicted in Figure 2 in comparison with the previously reported growth QTLs detected in this intercross (11). QTL graphs for loci detected on chromosomes 1, 3, and 7 are given in Figure 3.

There was a highly significant correlation between body weight at slaughter (i.e. 70 d) and body composition traits (Table 2). Therefore, body weight at 70 d was included as covariate in the QTL analysis to allow us to detect differences in body composition at a fixed weight. Family and sex were included in the model for those traits where a significant effect of family or sex was detected by the ANOVA (Table 1).

Abdominal fat deposition. We detected three suggestive QTLs for abdominal fat content (Table 5). This is marginally higher than the single suggestive QTL expected to occur as a Type I error in a full genome scan. However, we believe that all three reflect true QTL effects because they are all co-localized with QTLs affecting other body composition traits and/or growth (Figures 2 and 3). For two of

the QTLs, the allele from the High line was associated with higher fat deposition.

Table 5. Summary of QTLs affecting correlated responses to selection for growth in chicken.

Trait (unit)	Chr.	Pos. (cM)	F^1	Additive effect± SE[2]	Dominance effect±SE[2]	Var(%)[3]	Marker1[4]	Marker2[4]
Abdominal fat (g)	1	438	7.3†	-0.83±0.22	-0.13±0.32	3.5	*LEI0162*	*LEI0134*
	3	125	5.7†	0.55±0.26	-1.3±0.5	2.8	*MCW0222*	*MCW0004*
	7	41	8.2†	0.85±0.23	0.57±0.35	4.0	ADL0169	*ADL0279*
Breast muscle (g)	1	19	6.5†	-3.0±0.8	-0.05±1.60	6.2	*MCW0168*	*MCW0254*
	1	467	10.0**	-5.4±1.2	1.0±3.2	9.2	*LEI0162*	*LEI0134*
	3	107	10.9**	-4.6±1.0	-2.4±1.9	9.9	MCW0222	*ADL0371*
	4	215	6.7†	0.96±0.73	-3.7±1.1	6.3	LEI0076	*MCW0098*
Shank (g)	1	460	30.4**	2.4±0.3	0.27±0.72	13.1	*LEI0162*	*LEI0134*
	26	0	6.7†	0.33±0.22	1.0±0.3	3.2	*LEI0074*	MCW0069
	27	5	14.2**	1.27±0.24	0.08±0.39	6.6	*MCW0076*	*MCW0292*
Lung (g)	3	151	6.7†	0.25±0.08	0.32±0.14	3.2	*ADL0371*	*MCW0224*
Bursa (g)	17	0	5.8†	0.11±0.03	0.01±0.05	2.8	*ADL0199*	*ADL0149*
	26	39	6.1†	-0.11±0.03	-0.05±0.05	2.9	*MCW0069*	*MCW0286*
Spleen (g)	10	15	8.1†	-0.13±0.03	-0.04±0.06	3.9	*MCW0228*	MCW0194
	11	43	5.8†	0.13±0.04	-0.09±0.08	2.8	ADL0123	ABR0037
Glucose (mg/dL)	20	45	7.3†	0.007±0.007	-0.058±0.016	1.8	MCW0119	*HCK*
	27	21	9.3*	0.004±0.005	0.032±0.008	2.3	*ADL0376*	*MCW0292*
Cholesterol (mg/dL)	3	114	6.4†	3.8±1.1	-0.65±1.93	1.6	*MCW0222*	*ADL0371*
	9	78	6.1†	-0.57±0.87	4.2±1.2	1.5	*ADL0219*	*MCW0134*
	20	60	6.6†	3.3±1.2	-6.2±2.6	1.7	*ADL0125*	*BMP7*
Triglycerides (mg/dL)	2	217	7.4†	0.17±0.05	0.16±0.08	1.9	*UMA2.080*	*MCW0234*
Insulin (microIU/mL)	1	25	8.1†	0.24±0.08	0.36±0.14	2.2	*MCW0248*	*MCW0254*
	2	23	7.2†	-0.42±0.12	0.26±0.30	1.9	*ADL0190*	*MCW0063*
IGF-I (ng/mL)	1	480	7.6†	-0.57±0.15	-0.21±0.43	2.4	*ADL0245*	LEI0134

[1] *F* statistic for the QTL and level of significance; ** Genome-wide 1% significance, * Genome-wide 5% significance, and † Suggestive 5% significance.
[2] The additive and dominance effects were defined as the deviation of animals homozygous for the high line allele or heterozygous, respectively, from the mean of the two homozygotes.
[3] The reduction in the residual variance (%) of the F_2 population obtained by inclusion of a QTL at the given position.
[4] Flanking markers for QTL intervals estimated by the one-LOD drop method (20).

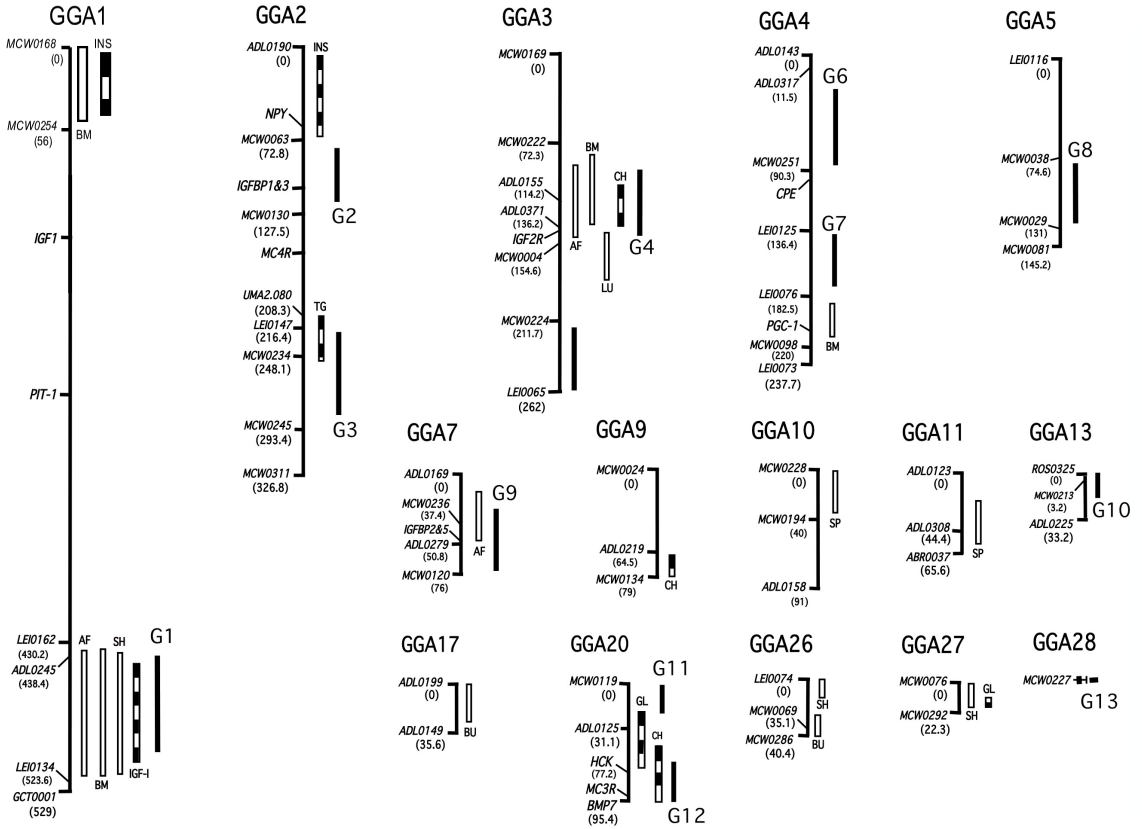

Figure 2. Schematic overview of QTLs detected in an intercross between the high and low growth chicken lines. Vertical open bars represent QTLs affecting body composition: AF-abdominal fat, BM-breast muscle, LU-lung, SP-spleen, BU-bursa, and SH-shank. Black and white bars represent QTLs affecting metabolic parameters: GL-glucose, CH-cholesterol, INS-insulin, TG-triglycerides, and IGFI-insulin-like growth factor I. Solid bars represent growth QTLs (11). Confidence intervals for the QTLs were estimated with the one LOD drop method (20). The closest flanking markers of each QTL and some obvious candidate genes are indicated. GGA1 = *Gallus gallus* chromosome 1, etc.; G1-G13 = *Growth1-Growth13* QTLs.

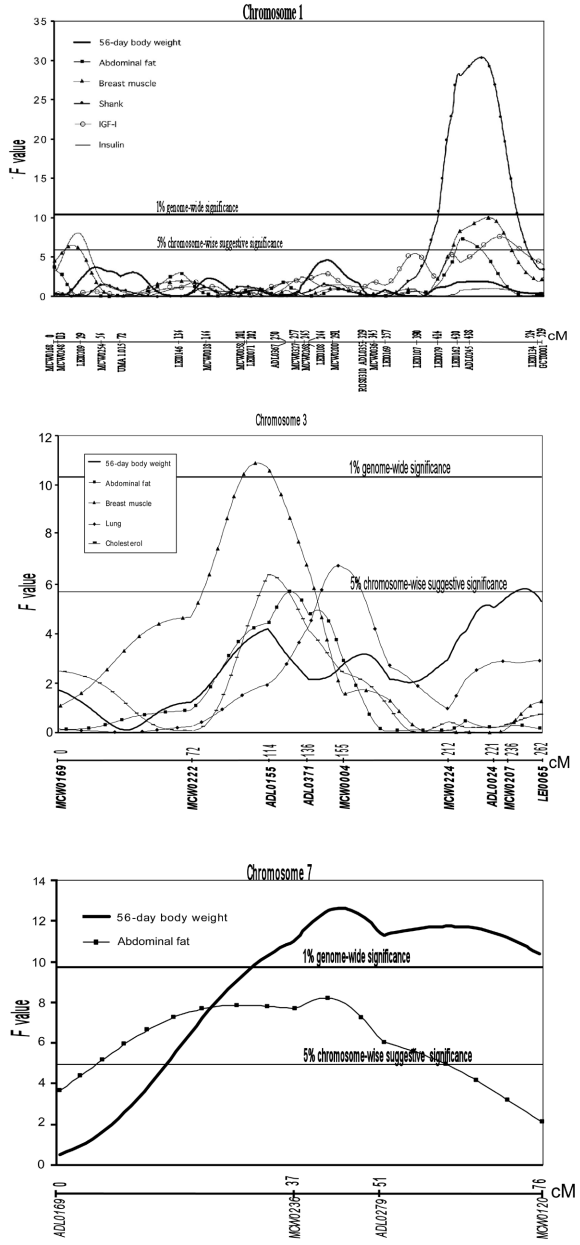

Figure 3. Test statistic curves for chicken QTLs detected on chromosomes 1, 3, and 7 using an intercross between the high and low growth selection lines. The marker map with distances between markers in Kosambi cM is given on the X-axis. The Y-axis represents the *F* ratio testing the hypothesis of a single QTL in a given position on the chromosome. The horizontal lines represent the 1% genome-wide and 5% suggestive significance thresholds.

Muscle mass. Two suggestive and two highly significant QTLs for breast muscle weight were detected (Table 5). The latter two were located on chromosomes 1 (around position 467 cM) and 3 (around position 107) and they explained 9-10% of the residual phenotypic variance when body weight at 70 days was used as covariate. The two QTLs were located in the regions harboring the *Growth1* and *Growth4* QTLs as well as QTLs for abdominal fat and shank weight. At both these loci the allele from the high growth line was associated with higher body weight but less breast muscle mass.

Shank weight. One suggestive and two highly significant QTLs for shank weight were identified (Table 5). The two highly significant QTLs on chromosomes 1 and 27 showed largely additive effects and explained 13.1% and 6.6%, respectively, of the residual phenotypic variance for this trait. At both loci the allele from the high line was associated with heavier shanks. The QTL on chromosome 1 was co-localized with *Growth1* and the QTLs for breast muscle weight and fat deposition described above (Figure 2). However, the QTL on chromosome 27 was not detected in our QTL analysis of growth. The suggestive QTL on chromosome 26 showed overdominance and it may or may not reflect a true QTL effect.

Weight of internal organs. We did not detect any convincing QTL for the weight of lung, bursa, or spleen (Table 5). We observed five suggestive QTLs for these traits compared with three expected to occur by chance only, when carrying out three genome scans. Only the one for lung weight on chromosome 3 was located in the vicinity of convincing QTLs for other traits (Figure 2).

QTL analysis of metabolic traits

The concentrations of glucose, insulin, glucagon, IGF-I, cholesterol, and triglycerides in blood plasma were measured when the birds were 63 days of age. QTL analysis of these six traits revealed one significant and seven suggestive QTLs which are only marginally higher than what we expect by chance (Table 4).

However, we think that several of these are true QTLs because as many as six showed a location that overlapped with QTLs for growth and/or body composition (Figure 2). The significant QTL for glucose on chromosome 27 showed overdominance; i.e. there was no significant difference between the two homozygotes whereas the heterozygote had significantly higher glucose values than the mean of the two homozygotes.

DISCUSSION

Forty-one generations of bidirectional selection for body weight at 56 days of age from a common base population resulted in dramatic differences in body weight and a number of correlated responses for body composition and metabolic traits between the high and low growth lines. Since the criterion of selection in these two lines across all generations has been solely for high or for low body weight at 56 days of age, we expected that the great majority of QTLs detected in our intercross between these two lines should influence body weight. The results are, in fact, in good agreement with this expectation. We detected 13 QTLs affecting growth that segregated in our reciprocal intercross (11). Most of the convincing QTLs for body composition and/or metabolic traits detected in the present study were located in the vicinity of growth QTLs (Figure 2). The only exception was chromosome 27 for which no growth QTL was detected in our previous study (11) but which harbored one highly significant QTL for shank weight and a significant QTL for plasma glucose concentrations. It is an open question whether these results represent one or two QTLs on chromosome 27, but a single QTL is less likely because the shank QTL showed perfect codominance whereas the glucose QTL showed overdominance. The clear trend for colocalization beween QTLs for growth and QTLs for correlated traits may be caused by pleiotropy or "linkage drag"; the latter means that a selection pressure on a QTL will influence the allele frequencies at closely linked QTLs affecting other traits. High resolution mapping is required to resolve whether any colocalization reflect pleiotropy or linkage (19).

How can we explain the observation of a highly significant QTL for shank weight at chromosome 27 with no significant effect on growth, despite the fact that the selection scheme was focused entirely on growth? Firstly, the QTL difference on this chromosome may have developed by genetic drift during the course of the selection experiment. This appears less likely since the observed effect of this QTL makes sense in relation to the phenotypic differences between lines. The QTL allele inherited from the high line was associated with heavier shanks that should be able to carry a heavier bird. Secondly, it could be a matter of statistical power. The main conclusion from our previous study (11) was that the difference in body weight between the high and low lines was determined by many QTLs each with a small effect. Many of the QTLs were on the border to reach the stringent statistical significance threshold that is required in a genome scan. Thus, the QTL on chromosome 27 may influence growth as well, but it did not reach the significance threshold in the analysis of growth. However, the previous QTL analysis did not indicate the presence of a growth QTL on chromosome 27, not even using a nominal significance threshold. Thirdly, the effect on growth

of this locus may have a threshold effect which means that it is only observed when the birds have reached a certain weight and most F_2 birds did not pass a putative threshold at which more robust shanks were required for high growth.

Another interesting observation was that the 13 growth QTLs detected in this intercross explained only 1.3-3.1% of the residual phenotypic variance for growth or body weight (11) whereas most of the significant QTL tests for body composition gave estimates of the explained residual variance higher than this and three were above 9%. Growth is a highly complex trait affected by many loci influencing appetite, feed uptake, nutrient allocation, body composition, metabolic rate, physical activity, etc. This means that any individual locus affecting growth in this cross explains only a rather small fraction of the genetic variance. In contrast, we expect that a more limited number of QTLs affects body composition and thus each one of them will explain a larger fraction of the variance for the correlated trait in the F_2 generation.

One of the more interesting observations in this study was the highly significant effects of reciprocal crosses on body weight at hatch, and on plasma concentrations of glucose, cholesterol, insulin, and IGF-I but with no significant effect on body weight at 56 days of age, the age at which selection took place. The reciprocal cross explains an astonishing 15-35% of the phenotypic variance for body weight at hatch, glucose, and insulin. F_2 chickens having a maternal grandmother from the high line were heavier at hatch, had higher glucose, cholesterol, and IGF-I concentrations but lower insulin levels. To interpret the cause of these effects one needs to consider the genetic constitution of the F_2 birds as regards mitochondrial DNA (mtDNA) and the sex chromosomes as outlined in Table 4. Thus, if the same effect is observed in both males and females it is likely to reflect a maternal effect or genetic differences in mtDNA. An effect only observed in females is likely to reflect differences in the W chromosome since the F_2 females are “balanced” as regards the Z chromosome. An effect only seen in males would most likely reflect the segregation of QTLs located on the Z chromosome. All the effects of reciprocal crosses showed essentially the same pattern in both males and females. Thus, they are most likely caused by either maternal effects or differences in mtDNA. A maternal effect appears to be a plausible explanation for weight at hatch since F_1 females that are offspring to a high line female rather than a low line female are slightly larger and it is well known that larger females produce larger eggs which in turn cause a larger hatch weight (26). A maternal effect appears less likely for the effects on metabolic traits which thus maybe caused by genetic differences in mtDNA. This is a possible explanation due to the key role of the mitochondria in energy metabolism. Interestingly, about 0.5% to 2.8% of all patients with Type II diabetes have mtDNA mutations (6). The question whether

the observed reciprocal cross effects are caused by maternal influence or mtDNA differences can be resolved using data from our forthcoming F_8 intercross generation. Six generations of intercrossing should have randomized any association between maternal effect and an effect caused by differences in mtDNA.

Although the confidence intervals for the observed QTLs are large we would like to point out some obvious positional candidate genes. The QTL regions on chromosome 3 and 7 harbor the genes for the insulin-like growth factor 2 receptor (*IGF2R*) and IGF-binding proteins 2 and 5 (*IGFBP2*, *IGFBP5*), respectively (Figure 2). The suggestive QTL for breast muscle weight on chromosome 4 maps to a region containing the gene for peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (PGC-1). Previous studies have shown that the nuclear PGC-1 protein is involved in the regulation of genes affecting energy metabolism as well as muscle physiology (13, 25). Lin et al. (15) showed that expression of PGC-1 is involved in control of fiber-type composition in mouse skeletal muscle.

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V

Evaluation of *MC3R* as a positional candidate gene for a growth QTL in chickens

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Summary

The melanocortin 3 receptor (MC3R) is involved in body weight control in mammals. Our previous QTL study using an intercross between high and low weight selected lines of chicken revealed a QTL for early growth on chromosome 20. *MC3R* is positioned within the QTL confidence interval and is thus a logical candidate gene for the QTL. Fine mapping of the region strengthened *MC3R* as a candidate gene, as the QTL peak has its highest value at this gene. Sequence analysis of the *MC3R* coding region revealed four nucleotide substitutions and the two lines were fixed for different alleles at this locus. This further strengthens *MC3R* as a positional candidate gene because we expect to observe pronounced allele frequency differences at those loci that have responded to selection. However, the four substitutions were all synonymous and are thus unlikely to have a causative role. *MC3R* expression in a brain region containing hypothalamus was studied using samples collected at hatch and at 56 days of age. *MC3R* expression was significantly lower in males than in females consistent with the strong sexual dimorphism in growth in chickens and the previous observation in mammals that *MC3R* expression tends to reduce body weight. Similarly, we observed a 50 to 90% higher expression at hatch in low line males and females compared with high line birds. The relative expression of *MC3R* alleles from the high line and the low line was

also studied within heterozygous F_1 individuals. A small (5%), but significant, increased expression of the low line allele compared to the high line allele was seen in cDNA compared to genomic controls. The results show that the differential expression is primarily due to trans-acting factor(s) but a cis-acting effect is also present.

Introduction

Quantitative Trait Loci (QTL) analyses in domestic animals have proven to be a successful approach to identify genes underlying complex traits (Van Laere et al., 2004; Andersson and Georges, 2004). We have recently detected a QTL for early growth on chromosome 20 using a large intercross between two selected lines in chicken showing a dramatic difference in body weight at 56 days of age (Jacobsson et al., manuscript). The melanocortin 3 receptor (*MC3R*) gene was identified as the major positional candidate located within the confidence interval of the QTL.

The melanocortin receptors belong to the G-protein coupled, seven transmembrane receptor family. The five identified melanocortin receptors have various functional roles, including regulation of skin and hair pigmentation as well as energy homeostasis and feed intake (Butler and Cone, 2002; McKenzie et al., 2003; Schioth et al., 1999; Valverde et al., 1995). *MC3R* and *MC4R* have been associated with body weight control in mammals (Dubern 2001, Cummings and Schwarz, 2000). Chen et al. (2000) showed that *MC3R* knock-out (-/-)-mice have reduced lean mass content, increased fat mass and higher feed efficiency compared to wild type mice. Furthermore, the body weight of *MC3R* and *MC4R* double knock-out mice were higher than either single knock-out mice. Altogether, there are strong indications that *MC3R* plays a role in body weight control in mammals and thus it is an obvious candidate gene for our growth QTL on chromosome 20. In this paper we report QTL fine mapping and *MC3R* expression analysis, which strengthen *MC3R* as a positional candidate for the growth QTL on chromosome 20.

Materials and methods

Animals

The high and low body weight lines used in this study have been developed by divergent selection for body weight at 56 days of age for more than 40 generations (Dunnington and Siegel, 1996). We recently crossed these lines to generate a large intercross pedigree for QTL mapping comprising 59 founder animals, 8 F_1 males, 75 F_1 females and 874 F_2 progeny (Jacobsson et al., manuscript). The founder lines as well as subsequent intercross generations are

maintained at Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

Microsatellite genotyping

Five microsatellite markers were developed in the *MC3R* region on chicken chromosome 20. The chicken genome sequence (February 2004 assembly) was used to design primers for the *HGEN002*, *HGEN003*, *HGEN004*, *HGEN006* and *HGEN009* loci (HGEN002F CAG GAC GTT GTA AAA CGA CTG TTT ATC TGA AGA CTA TTA GCA TGA GA, HGEN002R ATT TTT CAT TTA TTC CTG TGT GC, HGEN003F CAC GAC GTT GTA AAA CGA CGA AAA GTG TTA ATC AGT GCG ACC CG, HGEN003R CGC AGA TGA CAC CAA AAG GAC CA, HGEN004F CAC GAC GTT GTA AAA CGA CGG GGC GGG GGA GAG AAG GT, HGEN004R TGC GGG TAC TGG AGC TGG GA, HGEN006F CAC GAC GTT GTA AAA CGA CGA TTC CCA CAG ACT GCC CCC CGT, HGEN006R TGC CCT GCA GCA TCG CCT CGG TCT, HGEN009F CAC GAC GTT GTA AAA CGA CTG CCA GGA GGG GAG GAT TTC ATT, HGEN009R TGG GAG CTA TTC CTT GC ACCT CG), comprising dinucleotide repeats. The forward primer of the microsatellite markers were tailed with the M13 sequence in order to facilitate amplification with fluorescently labelled M13 primer (Schuelke, 2000) and thus increase the possibility for multiplex analysis. For PCR, a total of 20 ng DNA was amplified in 10 µl PCR reactions containing 1X PCR buffer (Applied Biosystems, Inc., Foster City, C.A.), 20 mM $MgCl_2$, 5 mM dNTPs, 0.5 U of AmpliTaq Gold (Applied Biosystems, Inc., Foster City, C.A.), 2 pmol of the M13-tailed forward primer and 10 pmol of the reverse and the fluorescently labelled M13 primer. PCR was carried out in a geneAmp PCR system 9700 (Applied Biosystems, Inc., Foster City, C.A.) machine and started by DNA denaturation for 5 min at 94^{o}C, followed by a touchdown PCR program starting with 94^{o}C for 30 s, annealing for 30 s and elongation at 72^{o}C for 30 s. The annealing temperature started at 65^{o}C and was decreased with one degree per cycle for 14 cycles, to 51^{o}C and an additional 35 cycles was run at constant annealing temperature. To ensure full length PCR products an additional elongation for 10 min at 70^{o}C was added after the last PCR cycle.

A MegaBACE instrument (Amersham Biosciences, Uppsala, Sweden) was used for fragment separation and the subsequent fragment analysis was done using the Genetic Profiler software. All genotypes were checked manually to minimize genotyping errors.

RNA isolation and cDNA synthesis

Tissue samples for RNA preparation were taken at 0 and 56 days of age in the high and the low line from generation 45. Samples from reciprocal F_1 intercrosses of the lines were also collected at 0 and 56 days of age. For each age, ten chickens from each line (5 males and 5 females) and 20 F_1 progeny (5 males and 5 females from each reciprocal intercross) were sampled. A brain region of the chicken containing diencephalon, mesencephalon, pons and medulla (DMPM) was dissected, immediately frozen in liquid nitrogen and stored at –70ºC until further analysis.

RNA was purified from the DMPM brain region**.** Each tissue sample was crushed and homogenized into powder in presence of liquid nitrogen, followed by extraction of total RNA with TRIzol® (Invitrogen, Frederick, MO, USA). RNA concentration and purity was measured using the Agilent 2100 bioanalyzer and Nanodrop instruments.

The RNA used for quantification of allelic expression was DNAse treated using the DNA-*free*™ kit (Ambion). cDNA was synthesized using random primers and the First-Strand cDNA synthesis kit (Amersham Biosciences). cDNA was purified with the Qiagen PCR purification kit (Qiagen). A previously published primer pair (Fitzsimmons et al., 2004) amplifying Ribosomal protein L14 (accession no AY579771) was used to check cDNA for DNA contamination. The product length is 145 bp for cDNA and 514 bp for DNA. PCR reactions were performed in a GeneAmp PCR system 9700 instrument. Two ul cDNA or 20 ng of genomic DNA was used in a 10 µl reaction containing 1 µl 1X PCR buffer, 30 mM $MgCl_2$,10 mM dNTPs, 5 pmol of each primer and 0.5 U AmpliTaq Gold. The touchdown PCR protocol described above was used.

RNA for real time PCR quantification was treated with RNase-free DNase (Promega, Madison, WI). One µg total RNA was used for cDNA synthesis with TaqMan Reverse Transcriptase reagents (PE Applied Biosystems) in a final volume of 50 µl (1xTaqMan RT buffer, 2.5 µM random hexamers, 500 µM each dNTP, 5.5 mM $MgCl_2$, 0.4 U/µl RNase inhibitor, and 1.25 U/µl Multiscribe RT). The reaction was performed for 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C.

Real-time quantitative PCR

One µl cDNA was used as template in the PCR reaction. Analysis of gene expression was performed using the SYBR Green I real-time PCR assay and the ABI PRISM 7700 Sequence Detection System

(PE Applied Biosystems). PCR reactions were done in duplicates with activation of AmpliTaq Gold DNA Polymerase for 10 min at 95°C and 40 cycles were run using two-step PCR (95°C/15 s, 60°C/60 s). As references, chicken _-actin and chicken glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used. Primers for chicken *MC3R* and the reference genes were designed with Primer Express 1.5 software (Applied Biosystems) (chMC3R F: 5'-CTT CCT CAT GGC CTC CCT TT-3'; chMC3R R: 5' GCT GCG ATG CGC TTC AC-3'; chActin F: 5'-AGG TCA TCA CCA TTG GCA ATG-3'; chActin R: 5'-CCC AAG AAA GAT GGC TGG AA-3'; chGAPDH F: 5'-GGG AAG CTT ACT GGA ATG GCT-3'; chGAPDH R: 5'-GGC AGG TCA GGT CAA CAA CA-3'). Each sample was assigned a CT (threshold cycle) value corresponding to the PCR cycle at which fluorescent emission, detected real time, reached a threshold above baseline. PCR products were gel-separated to confirm a band of the expected size. Data were normalized against the reference gene expression level and against the tissue weight to remove dilution effect of *MC3R* mRNA that may be expressed in a limited brain region.

Quantification of allelic expression

A pyrosequencing test was developed to quantify the expression of *MC3R* alleles from heterozygous F_1 individuals. Primers MC3R_EXP2_Bio (CAT CTG GAT CTC CTG CAT CAT) and MC3R_EXP2_R (AGG GAG GCC ATG AGG AAG AGC) was used to amplify a 117 bp fragment of *MC3R*. The forward primer was labelled with biotin. The PCR reaction was carried out in 10 µl reactions containing 1X PCR Buffer, 20 $MgCl_2$, 5 dNTPs, 0.5 U AmpliTaq Gold and 4 pmol of each primer. The reactions were run in a geneAmp PCR system 9700 machine starting with 94^{o}C for 5 min to denaturate the DNA, followed by 35 cycles of 30 s at 94^{o}C, 30 s at 52^{o}C and 30 s at 72^{o}C. The program ended with an additional 10 min of elongation at 72^{o}C.

Standard pyrosequencing protocols were used for the preparation and capture of PCR products on streptavidin-coated beads prior to pyrosequencing. The sequencing primer MC3R_EXP_SEQ (GAC AGT TTT GCT TTC) was used. A minimum product height of 1300 units was required for accurate allele quantification. cDNA and genomic DNA from 20 F_1 individuals were analysed. Duplicates of F_1 cDNA and DNA samples were simultaneously amplified and analysed in the pyrosequencing instrument. The experiment was performed twice to investigate the level of variation between PCR runs. This resulted in four (two duplicated) measurements of each individual sample. For each sample, a mean relative expression over all four measurements was calculated.

Statistical analyses

The CRI-MAP software (Green, 1990) was used for linkage analysis. The "BUILD" and "FLIPS" options were used to construct and evaluate the order of linked loci. QTL analysis was performed using the interval composite mapping method with the internet-based QTL Express software (Seaton et al., 2002). Family and sex were used as fixed effects in both-sex analyses and family in sex-specific analyses. Fst values were calculated using the Fstat software version 2.9.3.2 (Goudet, 2001). The Fstat software calculates Fst-values as proposed by Weir and Cockerham (1984).

Results

Fine mapping of the QTL region

Three new microsatellites from the *MC3R* region were developed based on the chicken genome sequence (February 2004) in order to fine map the previously identified QTL for body weight at 14 days of age. The markers *HGEN002*, *HGEN003* and *HGEN004* were genotyped in our F_2 mapping population and the linkage analysis confirmed the order of the loci as given in the genome assembly. The map order *HGEN004-HGEN003 -MC3R-HGEN002* was supported with a LOD score of 7.2 over the second best order.

The analysis revealed a QTL predominantly expressed in males and with strongest statistical support over *MC3R* (Figure 1). When comparing QTL results including family as fixed effect with those achieved excluding the family effect, a clear drop in significance level was seen for the joint-sex analysis (F from 12.0 to 7.3) resulting in the QTL failing to reach 1% genome-wide significance. However, for the sex-specific analyses the results are less dependent on the family effect (F from 10.4 to 10.9 for males and 2.4 to 0.6 for females). Thus, the male-specific effect reaches genome-wide significance independent of the inclusion of the family effect in the statistical model.

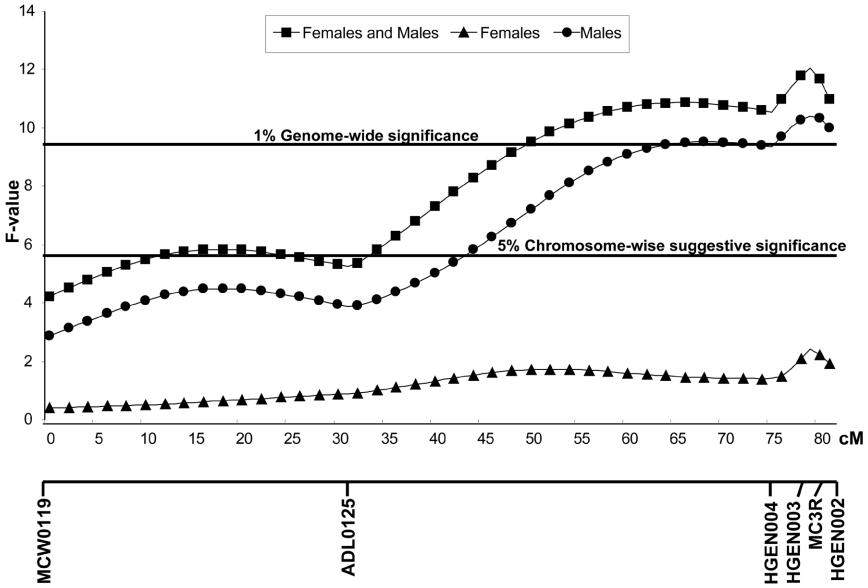

Figure 1. QTL graph for body weight at 14 days of age based on an intercross between the high and low weight chicken lines. The results of a QTL scan involving both sexes as well as a sex-specific analysis are shown. The linkage map for chromosome 20 is shown below the graph.

The high and low weight lines are fixed for different MC3R alleles

The entire *MC3R* coding region (a single exon) was determined from two individuals of each line. The sequence comparison revealed four line-specific SNPs (Table 1). All four were synonymous substitutions. The SNP at nucleotide position 894 was genotyped in all founder animals using the pyrosequencing-based test as previously described (Jacobsson et al., manuscript). This showed that all high line birds were homozygous for the *T* allele whereas all low weight birds were homozygous *C/C*.

Table 1. *MC3R* single nucleotide polymorphisms (SNPs) in the high and low weight chicken lines.

	Nucleotide position			
Line	549	564	882	894
Low weight	G	C	A	T
High weight	A	T	G	C

Based on the strong selection pressure for high body weight, we expected the lines to be fixed or close to fixation for different haplotypes at chromosomal regions harbouring a causative mutation underlying a QTL controlling body weight. We therefore developed two additional microsatellites (*HGEN006* and *HGEN009*) which

were used together with other markers from this region of chromosome 20, including *MC3R*, to study the allele frequency distribution in the two lines. For each locus we calculated Fst values, to estimate genetic divergence between lines, and the expected degree of homozygosity within each line (Figure 2). We found that the weight lines are fixed for different *MC3R* alleles, but not for any of the flanking markers. The two closest flanking markers, *HGEN002* and *HGEN003*, are also fixed in the low line but they are still segregating in the high line (Figure 2).

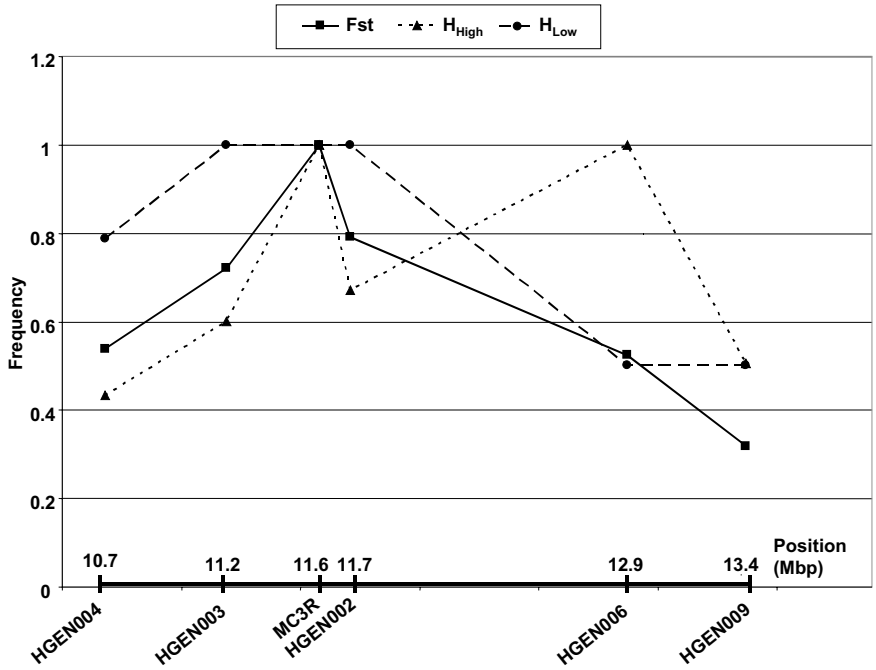

Figure 2. Graph illustrating allele frequency differences in the high and low weight chicken lines across a 2.7 Mbp region around *MC3R*. Fst values and expected homozygosity are given.

Expression analysis

Two different methods were used to measure *MC3R* expression. Expression differences between the parental lines were measured by real-time quantitative PCR in relation to the house-keeping genes *GAPDH* and *β-actin*. Pyrosequencing was used to quantify the relative expression of the high and low line alleles in heterozygous F_1 individuals. Expression was studied in a brain region enhanced for hypothalamus and containing diencephalon, mesencephalon, pons and medulla (DMPM).

At hatch, there were a 1.8-1.9 fold higher MC3R expression in the low line compared with the high line using *GAPDH* as control (Table 2; Figure 3). The result obtained with _*-actin* confirmed the result but the fold change was slightly lower (1.4-1.5). However, there was no clear differential expression between lines at 56 days of age (Table 2; Figure 3).

Table 2. *MC3R* expression relative to *GAPDH* and *ß-Actin* in the high and low weight chicken lines. Expression was measured in a brain region containing hypothalamus and at two ages, at hatch and at 56 days of age. The ratio of the expression in the low line versus the high line is given.

Age	n	*GAPDH*	*ß-Actin*
0 days, male	5	1.8**	1.4*
0 days, female	5	1.9*	1.5
56 days, male	5	1.0	0.8*
56 days, female	5	1.3	1.1

*P<0.05; **P<0.01

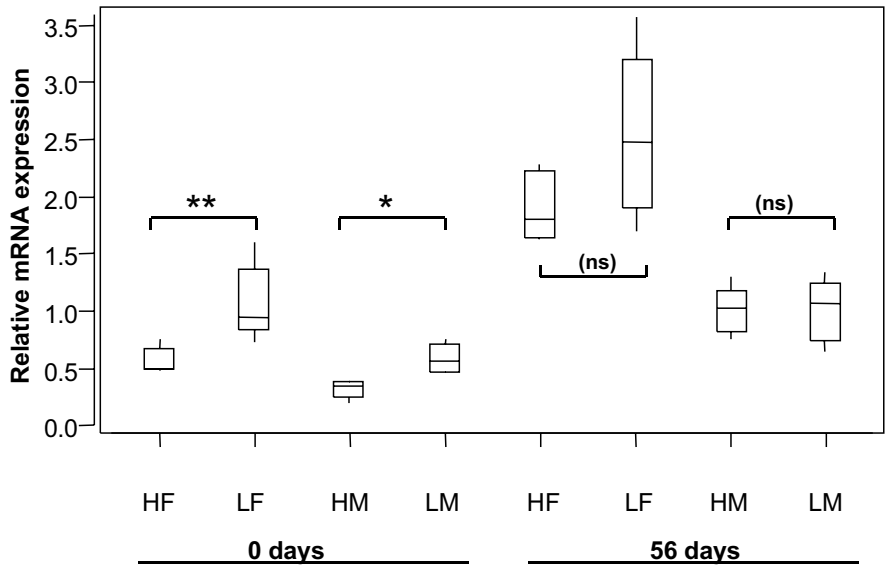

Figure 3. Boxplots of *MC3R* mRNA expression relative to *GAPDH* expression in chicken lines divergently selected for high (H) and low (L) body weight at 56 days of age. Relative expression was measured at 0 and 56 days. M=male. F=female.

Pyrosequencing was used to quantify the relative expression of *MC3R* alleles inherited from the high and low line alleles in cDNA samples from heterozygous F_1 individuals. We could not design a *MC3R* RT-PCR amplicon that spanned an exon/intron border because *MC3R* contains a single exon. RT-PCR analysis using our reference sequence (Ribosomal protein L14) showed that our DNAse

treated RNA contained no or only a minor amount of contaminating genomic DNA. Pyrosequencing was done using brain cDNA samples from 20 F_1 birds (equal number of males and females from each reciprocal cross). The results revealed that 19 birds were heterozygous *C/T* at nucleotide position 549 (reverse strand), as expected, whereas one was homozygous *T/T*. The latter shows that the *C* allele is not completely fixed in the low line. Quantification of the pyrograms revealed no allelic imbalance using genomic DNA but a 5% higher expression of the *C* allele from the low line using cDNA (Table 3).

Table 3. Relative quantification of the *MC3R* allele inherited from the low line versus the high lines using cDNA samples and genomic DNA.

Group	N	Mean±SD
cDNA	19	1.05±0.001
Genomic DNA	20	1.00±0.001

The difference in allele quantification in cDNA and genomic DNA is statistically significant in a two-tailed t-test ($t=2.53$, $d.f.=36$, $p<0.016$); there was no statistically significant effect of sex or of reciprocal cross.

Discussion

MC3R is an obvious candidate gene for our previously described QTL affecting early growth on chicken chromosome 20 (Jacobsson et al., manuscript). In the present study the region was further studied by using an additional three microsatellite markers near *MC3R* in the QTL analysis. The statistical evaluation revealed a highly significant male-specific QTL with its highest peak close to *MC3R* (Figure 2). Sex-specific analyses for body weight at other ages (0, 28, 42, 56 and 70 days of age) or metabolic traits (plasma concentrations of glucose, insulin, glucagon, IGF1 and triglycerides) did not reveal any QTL effects that were statistically significant using genome-wide thresholds.

The lines are expected to be fixed for different haplotypes at those major QTLs that have responded to the strong divergent selection for high and low body weight. The size of the haplotype affected by a selective sweep depends on the local recombination rate and how fast the causative mutation became fixed in a given line. We observed that the lines are fixed for different *MC3R* alleles, but not for the flanking markers situated only 400 kb and 100 kb on either side of the gene. Although, the flanking markers are fixed in the low weight line, they are still segregating in the high line. Thus, if the chromosome 20 QTL

is located in the region between 10.7 to 13.4 Mbp, as suggested by the QTL graph, the causative mutation is likely to be in the interval 11.2 – 11.7 Mbp, containing *MC3R*, where we observe the most pronounced allele frequency difference between lines. However, further markers need to be added in the 45 cM gap between *ADL0125-HGEN004* to ensure that the QTL peak is distal to *HGEN004*. We are maintaining an Advanced Intercross Line (AIL) of the high and low line, and we recently collected phenotypic data and genomic DNA from 400 F_8 birds. This will be a very important resource for the high resolution mapping of the *MC3R*-linked QTL.

In order to investigate whether differential expression of *MC3R* could be causing the QTL effect, we set out to measure the relative expression of *MC3R* in the selected lines. *MC3R* expression has previously been shown in brain in mammals and particularly in hypothalamus (Desarnaud et al., 1994; Roselli-Rehfuss et al., 1993). We sampled a brain region enriched for hypothalamus, but also containing diencephalons, mesencephalon, pons and medulla. Takeuchi and Takahashi (1999) have studied *MC3R* expression in various tissues in chicken and reported expression in the adrenal gland but no expression in brain. They used total brain for the expression study, which may have diluted the expression in specific brain regions down to an undetectable level. We measured relative expression of *MC3R* by real-time quantitative PCR and clearly revealed expression in the chicken brain. Based on previous work in mammals we expected low *MC3R* expression to be associated with higher weight (Cummings and Scwartz, 2000). Thus, the significantly lower *MC3R* expression in males both at hatch and at 56 days of age is entirely consistent with the strong sexual dimorphism for growth in chicken. Furthermore, the significantly lower *MC3R* expression in the high line at 0 days of age may be causally related to the enhanced growth in this line. Real-time PCR analysis indicated a 50 to 90% decrease in *MC3R* expression in both males and females from the high line compared with low line birds. No consistent difference in *MC3R* expression was observed at 56 days of age.

We sampled the same region of the brain for RT-PCR analsyis from 20 F_1 birds originating from reciprocal F_1 intercrosses to further investigate the basis for the observed differential expression of *MC3R*. A pyrosequencing test was developed to quantify the relative expression of the allele inherited from the high and low weight lines in heterozygous F_1 individuals. No normalisation to house-keeping genes is needed when using F_1 individuals as the F1's have an inbuilt internal control (the other allele). This experimental design allowed us to determine whether the observed differential expression is caused by trans- or cis-acting factors. We observed a small (about 5%), but statistically significant, difference in the expression level between alleles within F_1 heterozygotes. Although the results went in the

same direction as observed in the founder animals (lower expression in the high line) the minor difference strongly suggested that the markedly higher expression of *MC3R* in the low line (+50-90%) is primarily caused by one or more trans-acting factors. This could, for instance, be another QTL encoding a transcription factor influencing *MC3R* expression. Interestingly, a recent study of this intercross has revealed a highly significant epistatic interaction between a QTL on chromosome 7 and the *MC3R*-linked QTL (Ö. Carlborg et al., in preparation).

The allelic imbalance in F_1 heterozygotes suggest that a cis-acting regulatory mutation affects *MC3R* expression although the biological significance may be questioned due to the minor difference (~5%) in expression levels. This observation does not exclude *MC3R* as a positional candidate gene for our QTL. There are several reasons why a cis-acting effect may have been underestimated. The ages sampled may not have been optimal and the effect may be much more pronounced in a subset of cells with a critical role in regulating growth or appetite. It is also possible that interaction with other QTLs may have blurred the picture because an F_1 individual is heterozygous at all major QTL, including the one on chromosome 7 discussed above. Finally, the putative effect of *MC3R* may not be mediated through differential expression.

The sequence analysis of the *MC3R* coding sequence revealed four synonymous substitutions between the alleles fixed in the high and low weight lines. None of these is expected to cause the QTL effect. However, the present study provides a strong impetus to sequence the entire *MC3R* gene, including the 5'- and 3'-UTR, and its flanking regions in the search for mutations that may underlie the QTL and/or cause the small cis-acting effect on gene expression.

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