Genetic Studies of Pigmentation in Chicken

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Domestic animals have been selected by humans for thousands of years, which have drastically altered their genetic constitution and phenotypes. In this thesis, several of the most important genes causing pigmentation differences between the wild red junglefowl (*Gallus gallus*) and domestic chickens have been identified. Pigmentation phenotypes are easily scored, and the genes underlying these phenotypes are valuable models to study gene function and gene interaction.

**Dominant white** colour is widespread among domestic chickens. The *Dominant white* allele specifically inhibits the expression of black (eumelanin) pigment and we identified several insertion/deletion mutations in the *PMEL17* gene causing the different phenotypes controlled by this locus. The *Silver* allele on the other hand inhibits the expression of red (pheomelanin) colour and is a genetic variant of the *SLC45A2* gene. *Silver* is the first pheomelanin-specific mutation(s) reported for this gene. An 8 kb deletion, including a conserved enhancer element, 14 kb upstream of the transcription factor *SOX10* is causing the *Dark brown* phenotype. This phenotype restricts the expression of eumelanin and enhances red pheomelanin in specific parts of the plumage. These three gene identifications have extended the knowledge about genes affecting melanocyte function.

Carotenoid-based pigmentation is of utmost importance in birds and other animals. The *yellow skin* allele in chicken allows deposition of carotenoids in skin and explains why most domestic chickens have yellow legs. We demonstrated that the yellow skin phenotype is caused by a tissue specific regulatory mutation in the gene for the enzyme beta-caroten dioxygenase 2 (*BCDO2*). This was the first identification of a specific gene underlying carotenoid-based pigmentation. Interestingly, the *yellow skin* haplotype was shown to originate from the grey junglefowl (*Gallus sonneratii*) and not the red junglefowl as expected, thus presenting the first conclusive evidence for a hybrid origin of the domestic chicken.

**Keywords**: chicken, pigmentation, eumelanin, pheomelanin, carotenoids, *Dominant white*, *PMEL17*, *Silver*, *SLC45A2/MATP*, *Dark brown*, *SOX10*, *yellow skin*, *BCDO2/CMO2*, domestication

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ISSN 1651-6206
urn:nbn:se:uu:diva-98426 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-98426)
Till min familj
List of papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals.


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Introduction

This year (2009) it is 200 years since the birth of Charles Darwin and 150 years since he published his famous book “The origin of species” or as it was originally named “On the origin of species by means of natural selection”\(^1\). Darwin introduced the idea that all living organisms have evolved by the course of natural selection, and he used domestic plants and animals as examples of this, thereby questioning the ideas of the time that all domestic races had possessed their own wild prototype\(^1\). The theories by Charles Darwin have been greatly questioned and even if the acceptance for his evolutionary theory is higher today there are still many non-believers in the world. In a Gallup poll from 2008, only around 15% of the people in the United States believed in evolution and that humans have developed over millions of years\(^2,3\). The evolutionary theory is much more accepted in Sweden and other Nordic countries\(^2\).

Gregor Mendel (1822-1884) is the founder of genetic science, and ever since Mendel’s pioneering studies of the mechanism behind inheritance in peas, colour phenotypes have been studied\(^4\). It is now more than 100 years since Mendel’s findings were rediscovered and the first studies of plumage colour inheritance in chicken were performed\(^5\).

There are several advantages working with domestic animals when investigating phenotypic variation compared to more commonly used model organisms such as mice and rats. Domestic animals have been selected by humans for thousands of years and thus represent an abundant collection of mutations that affect their phenotypic traits\(^6,7\). Some of these traits are inherited according to Mendelian principles and caused by variation at a single locus, i.e. monogenic traits. Examples of monogenic traits are for instance the above mentioned colour phenotypes and simple familial heritable disorders. Other phenotypes are more complex and influenced by alleles at many loci as well as environmental factors. These phenotypes are called polygenic, complex or quantitative. Examples of complex traits are weight and behaviour.

In this thesis, phenotypic variation in plumage colour has been studied with chicken as the main model organism. It has been shown that colour loci in domestic animals have been selected for non-camouflaged patterns by humans, with the purpose of making it easier for the early animal farmers to keep control of their animals\(^8\). Colour phenotypes must also have been selected just because of the thrill of having something novel. With time some
of these skin and plumage colours have become of economic significance to the poultry industry. Different communities prefer different colours of the skin of their chicken, and some plumage colours are used to determine the sex of day old chicks for layer production\(^9\).

For a molecular geneticist the reason for studying pigmentation genetics is the considerable significance it can have in understanding gene function and gene interaction. Since some colour phenotypes are results of regulatory mutations, these studies can shed light on transcriptional regulation. Much more is to be learnt about the genetics underlying pigmentation and melanocyte development. In a broader perspective, studies like these could be of importance for both human and veterinary medicine. As an example, the Grey allele in horses predisposes for melanoma and the genes found to cause the Grey phenotype are obvious candidates for human studies\(^{10}\).

### Genetic and genomic studies of phenotypic traits

#### Genetic variation

In 1953 Watson and Crick published the structure of the DNA (deoxyribonucleic acid) double helix\(^{11}\). The building-blocks of DNA are the four nucleotides A (adenine), T (thymine), G (guanine) and C (cytosine). Triplets of these four nucleotides constitute the genetic code that is a feature shared by all living organisms. The size of the human genome is approximately 3 Gb (3,000,000,000 bp) and a single bp variation at the wrong position in the genome can be deleterious for the individual. Despite this fact, genetic variation is high and there is a lot of non-deleterious variation to be found. This variation is used as a tool by geneticists to determine the degree of genetic variation between individuals and classify them into groups depending on their DNA sequence.

The most common (and commonly used) type of genetic variation is SNPs (single nucleotide polymorphisms). SNPs are polymorphic nucleotides in the DNA sequence, for example an A to G change. Other common variations are short insertion/deletions (indels) of a few bp, and polymorphic di-, tri- and tetra-repeats (such as (AG)\(_n\)) called microsatellites. CNVs (copy number variations) are longer (>1 kb) segments of DNA variation, such as insertion/deletions or duplications.

Today there are many methods for SNP scoring and the method of choice depends on the number of markers used. When performing linkage studies, a large number of SNPs are required. Up until a few years ago, microsatellites were the genetic marker type chosen for this type of studies, but the fast development of technology for cost effective and accurate typing of hundreds to thousands of SNPs has quickly made microsatellite typing a method of the past. Today the cost for resequencing parts of or whole genomes is
rapidly decreasing, and perhaps will we in a few years time also see SNP typing as a historical method?

A phenotype is any observable trait of an individual and in this thesis monogenic colour phenotypes have been studied. During sexual reproduction one haploid set of chromosomes is inherited from each parent. At each polymorphic site in the genome the resulting individual can either become homozygous or heterozygous.

Alleles causing a distinct phenotype can be acting in a recessive, dominant or co-dominant fashion. For a recessive trait locus, two copies of the mutant allele are required to express the phenotype. For a dominant phenotype a single mutant allele inherited from one of the parents is sufficient. Some traits show co-dominance, with the heterozygous individuals being an intermediate between the two homozygous phenotypes.

When the phenotype is determined by a locus on the sex chromosomes the pattern of inheritance depends on the sex of the individual. The heterogametic sex (the one having two different sex chromosomes, male humans are XY, female birds are ZW) will always express the phenotype associated with an allele regardless of its recessive or dominant nature since the heterogametic sex only inherited one X (or Z) chromosome.

Linkage analysis

Linkage mapping can be used for the identification of chromosomal regions harbouring a gene/genes underlying a certain phenotype. An informative pedigree material and polymorphic genetic markers are required for linkage analysis. When two markers show a tendency of co-segregation they are in linkage with each other. A LOD (logarithm of the odds) score gives the odds of linkage for a marker to the phenotype investigated, and is calculated as the log (base 10) of the odds that two investigated loci are linked rather than unlinked. A LOD score of 3 is usually considered as significant evidence of linkage (odds in favour of linkage 1000:1). The recombination fraction (\( \Theta \)) is the number of recombinants divided by the total number of informative meioses and ranges from \( \Theta = 0 \) for loci that show complete linkage to a maximum of \( \Theta = 0.5 \) for loci showing independent segregation. A recombination fraction of 0.5 mean either that the loci are far apart on the same chromosome, or that they are located on different chromosomes. The map distance between two loci is given in centi-Morgan (cM). One cM is defined as 1% recombination between two loci\(^2\).

Pedigrees

To study the inheritance of a phenotype, a family material or pedigree is required. In human genetics several different families with the same disorder is usually vital to obtain a significant LOD score and thereby linking the
disease to a specific chromosomal region. When working with model organisms many more offspring can be generated (up to thousands if required). To initiate a pedigree, parental animals (P) with different phenotypes are crossed to generate a heterozygous F1 generation. These F1 individuals can then be intercrossed to generate an F2 generation segregating for all loci explaining phenotypic differences between the parental populations. When chromosomes are inherited from the F1 to F2 generation, recombination between the parental chromosomes occurs. The power of performing linkage analysis in large F2 populations is seen by the highly significant LOD scores generated. As an example the linkage between the Dominant white (I) locus and the PMEL17 gene studied in paper I resulted in a LOD score of 107.2! Another way to generate a pedigree is to backcross the F1 animals to one of the parental populations.

Positional cloning and identification of the causative mutation

After linking the phenotype of interest to a chromosomal region the next step is to narrow down the region and identify candidate genes and the causative mutation. In fine-mapping experiments, more genetic markers are added in the region of interest. The resolution of the region depends on the recombination frequency in the region and the size of the pedigree used for mapping.

When the region has been reduced to its limit by linkage analysis using a pedigree material, no more recombination events can be found between the closest markers and the investigated trait locus. The next step is to find the minimum shared haplotype that is identical by descent (IBD) between individuals carrying the same ancestral mutation causing the phenotype. In an experimental cross, intercrossing the F2 animals to generate subsequent generations, with recombination occurring in every generation, can generate such a material. Another approach is to genotype or sequence animals from other breeds sharing the phenotype of interest, under the assumption that they carry the same causative mutation.

In paper III of this thesis we sequenced animals with different alleles at the studied locus. This resulted in an IBD region among the animals with the mutated Dark brown phenotype, but also gave us the causative mutation since one of the wild-type individuals carried the ancestral haplotype. To sequence animals from many different breeds with and without a causative mutation has shown to be useful before. By sequencing several animals with and without a QTL (quantitative trait locus) allele affecting muscle growth, Van Laere et al. 2003 found a regulatory nucleotide substitution to be the causative mutation, QTN (quantitative trait nucleotide) for a major QTL in pigs13.

If there are many possible causative mutations in an IBD region several web-based tools can be used to find candidate genes as well as regulatory regions. Thanks to the many sequenced genomes, information about genes,
conservation and variation between species (and individuals), and much more can be accessed with the help of several web browsers, for instance the UCSC browser (www.genome.ucsc.edu/). To be functional, a causative mutation within a gene usually changes a well conserved amino acid, deletes an exon(s) or generates a stop codon. Within regulatory regions functional mutations can be of several types: single base pair mutations or insertion/deletions resulting in gain or loss of binding sites, insertion/deletions can also affect the copy number of a given regulatory site. Regional duplications can result in novel regulatory regions and translocations can bring genes into the near vicinity of new regulatory domains. Sometimes new regulatory target sites can be induced by a single base pair change in what seems to be “non-functional DNA”. In the study of a QTL underlying muscle mass in sheep, a single base pair mutation in a non-conserved region was shown to create a microRNA target site, which resulted in a significant phenotypic effect.

Expression studies

If the phenotype is supposed to be caused by a regulatory mutation there are many ways to analyze this further. An analysis of samples from individuals with different phenotypes can show an expression difference at the mRNA level by various methods. Some of the most commonly used methods are Northern blot, qPCR or in situ hybridization.

In paper IV of this thesis a complement to these methods was used. The pyrosequencing method was here used to quantify the expression of the BCDO2 mRNA (cDNA) in liver and skin from individuals heterozygous for a SNP in the BCDO2 gene. This is an efficient method to quantify the relative expression of a wild-type and mutant allele. Another useful method to study the difference in expression is to perform direct sequencing on genomic DNA and cDNA from individuals heterozygous for the studied mutation, as seen in paper II of this thesis and also in the study of the Grey locus in horses.

Phylogenetic trees

A phylogenetic (evolutionary) tree attempts to reveal how different species (or breeds) are related to each other. In 1859, Charles Darwin presented the first “diagram” (tree) attempting to explain this. To root a tree an out-group is used. This is usually a species that is clearly more distantly related to the species under analysis. To draw correct conclusions from a phylogenetic tree analysis the input data must be well designed. For instance, hybridization between species can confuse the output. To show a consistent result of high quality, sequences from several regions of the genome (or whole genomes) should be used. By studying sequences from different parts of the chicken
Comparative genomics

Comparative genomics is the study of sequence and genome similarities found between species. Major methodological advances in sequencing technology and genome assembly during the last years have resulted in a rapid increase of sequenced genomes. Comparison of genome sequences from species that are closely or distantly related can answer different questions. By comparing as distantly related species as human and chicken, conserved elements important for essential biological functions can be identified. These sequences called MSCs\(^6\) (multispecies conserved sequences) include both genes and non-coding functional elements that are involved in controlling gene expression. When comparing closely related species much more of the sequence will be conserved and it can be difficult to find the functional elements specific for their short branch. To identify for example primate specific sequences numerous different species would be needed\(^7\). In conclusion, the shorter the branch length, the more species are required to be sequenced to find the MSCs specific for that branch, but to find some of the MSCs important for basic vertebrate functions just a few sequences from more distantly related species are required. The field of comparative genomics is rapidly growing and studies of functional non-coding elements will probably be the next big advance in the understanding of biological functions and causes for disease.

A simple way to access information on conservation between species is to use the publicly available genome browsers, for instance the UCSC Genome Browser (www.genome.ucsc.edu).

The comparison of genome sequences within a species can be used to find the functional variants involved in disease, but comparison to genome sequences from other species may be required to assess their biological significance.

In paper III of this thesis an element conserved between chicken, mouse and rat was found to be deleted, causing the \textit{Dark brown} phenotype.

Domestic birds as model organisms

Domestication of the chicken

Domestic chickens belong to the genus \textit{Gallus} that includes four (sub) species; the red junglefowl (\textit{G. gallus}), the Ceylon junglefowl (\textit{G. lafayettii}), the grey junglefowl (\textit{G. sonneratii}) and the green junglefowl (\textit{G. varius}).
Today the chicken is primarily used as a meat and egg producer but the history of chicken goes back a long time, and initially the domesticated chickens were used for religious purposes and cockfighting\textsuperscript{18}. The red junglefowl was domesticated several thousand years ago and has by many been thought to be the sole ancestor to the domestic chicken. Hutt\textsuperscript{19} wrote in 1949;

“…constant repetition of the familiar statement that all domestic fowls are descended from the Red Jungle Fowl of India has apparently led some writers to consider the question settled.”

In 1996 Fumihito et al. studied part of the mitochondrial (mt) DNA from different junglefowl species and concluded a monophyletic origin of the domestic chicken\textsuperscript{20}. Nishibori et al. (2005) questioned this conclusion when examination of the mtDNA and two segments of the nuclear genome from the different species of junglefowl indicated interspecies hybridization between red and grey junglefowls, and between grey and Ceylon junglefowls\textsuperscript{21}.

In his book from 1949, Hutt\textsuperscript{19} discussed the possibility that some of the colour phenotypes seen in domestic chickens might have been inherited from some other species than the red junglefowl, thus questioning the monophyletic origin of the domestic chicken. In paper IV we show that the yellow skin ($W^*Y$) allele originates from the grey junglefowl, confirming the hypothesis of Hutt.

Chicken as a model organism and the chicken genome

Chickens have been used as model animals to answer questions about development for thousands of years\textsuperscript{22}. By opening eggs of chicken and studying the progression of development at different stages, the Greek philosopher Aristotle (384 BC-322 BC) funded the theory of epigenesis (the development from a simple to a more complex organism). These types of studies are still an advantage with using chicken as a model animal in comparison to mouse. The chicken embryos develop outside the body of the mother and are therefore easily accessible, studied and manipulated.

Studies in chickens have among other things been important for studies of cell migration. The neural crest (NC), that generate associations between many different tissues and organs in the vertebrate body, is one of the structures of the embryo that has been studied in chicken during the last century, to a large extent thanks to the quail-chick chimera system\textsuperscript{23}. In these chimeras the heterochromatin is differently distributed in the nucleus depending on its ancestry and can be stained by different methods to study the actions and fate of the grafted cells. Studies in chicken have also contributed to various other fields, for example immunology, virology, cancer and genetics\textsuperscript{22}.

Genetic studies of chickens have been carried out for more than 100 years and plumage colour in chicken was one of the first traits examined after
Mendel’s initial studies. The Dominant white (I) phenotype in chicken was investigated already in 1902 and in the following years many more colour phenotypes were studied, for example Silver (S) in 1912. The size of the chicken genome (1 x 10^9 bp) is approximately one third of the human genome. Chickens have 38 pairs of autosomes and one pair of sex chromosomes. Most bird karyotypes (including chicken) have chromosomes of remarkably different lengths, referred to as macro- and microchromosomes. Females are the heterogametic sex (Z/W) while males are homogametic (Z/Z).

In 2004, the chicken genome sequence was published. It was a 6.6 x coverage draft sequence from a female red junglefowl. In addition to the red junglefowl genome sequence, 0.25 x coverage of the genome was also generated from a broiler, a layer and a silky (the sequenced layer was the hen Agda from the SLU13 line, Uppsala). From this study, 2.8 million SNPs in the chicken genome were identified with an average rate of about five SNPs per kb. The chicken recombination rate is high. In this investigation it was found to be 2.5-21 cM per Mb, with a higher recombination rate on microchromosomes, compared with ~1cM per Mb in humans.

Japanese quail

Japanese quail (Coturnix japonica) was domesticated around 1000 years ago. It was first used as a songbird but the popularity of the bird for this characteristic was reduced after World War II. Since domestication it has also been used for meat and egg production but never to the same extent as the chicken.

Later it also became popular as a laboratory animal. Japanese quail is a suitable experimental animal due to its small body size and short generation time (four months). The quails are also easy to handle and it is possible to keep many birds in a relatively small space. The disadvantage is that there are few breeds (varieties) of Japanese quail and the number of reported mutations in quail is small compared to chicken. Minimal selection has been carried out in quail, resulting in much less focus on the bird itself with regard to phenotypic anomalies and studies of mutants. Japanese quail is also very susceptible to inbreeding depression.

The plumage of wild-type adult Japanese quail is brown in variable shades, including parts of other colours ranging from black to creamy white. Around 20-30 different colour mutants have been found in Japanese quail, but only a few of them are available today. Some of the mutants reported in different studies are also alleles at the same locus and the same mutant phenotype has occurred several times. In paper II of this thesis two of these phenotypes were studied.
The development of pigmentation

The field of pigmentation biology is extensive. This summary will introduce the genes and pathways relevant for this thesis.

Melanocyte development

Melanocytes (pigment cells) originate from the neural crest. The neural crest consists of a group of multipotent embryonic cells that initially can be found at the dorsal side of the neural tube\(^\text{31}\). After widespread migration in the developing embryo many different cell types (melanocytes, neurons and glial cells, endocrine cells and mesenchymal cells) are derived from the neural crest\(^\text{31}\). Many proteins and pathways are involved in melanocyte specification. Transcription factors such as TCF/LEF work together with PAX3 and SOX10 to express the microphthalmia-associated transcription factor (MITF) that has a crucial role in melanocyte development\(^\text{32-34}\). Other pathways involved in the melanocyte development can also regulate MITF expression\(^\text{33}\). MITF itself regulates the expression of many genes important for melanocytes, among them \(\text{TYR, TYRP1, TYRP2 (DCT), PMEL17, SLC45A2 (MATP, AIM-1)}\) and \(\text{MC1R}\(^\text{33}\). An interspecies difference between mice and zebrafish in the regulation of the melanocyte development has been seen. The expression of \(\text{TYR}\) and \(\text{DCT}\) has in addition to the regulation by MITF, been shown to be directly regulated by SOX10 in mice, but in zebrafish there is a simple regulatory chain with SOX10 regulating MITF that in turn regulates the downstream targets\(^\text{33, 35-37}\) (Figure 1).

![Diagram of melanocyte development](image)

Figure 1. A simplified illustration of the factors that work together to regulate the expression of MITF and some of the genes that are under MITF regulation. The illustration also shows the direct regulation of DCT and TYR by the SOX10 transcription factor that has been documented in mouse. (Figure modified from Goding 2000\(^\text{32}\) and Hou and Pavan 2008\(^\text{33}\)).
SOX10

The SOX10 gene encodes a transcription factor belonging to the SOX (Sry-related HMG box) family group E. The SOX proteins control a variety of developmental processes including the melanocyte formation during neural crest specification\textsuperscript{31, 34, 38}. In 1998, a single base insertion resulting in a translation frameshift of SOX10 was found to be the causative mutation in the \textit{Dom} mice\textsuperscript{39, 40}. 	extit{Dom/ Dom} homozygous mice are embryonic lethal and heterozygous individuals have white spotting and defects in the colon\textsuperscript{41}. Another mouse phenotype, the \textit{Hry} mouse is associated with a 15.9 kb deletion of highly conserved sequences upstream of SOX10. The homozygous \textit{Hry} mouse shows a loss of melanocytes and a constriction of the colon (megacolon) resembling the Waardenburg-Shah syndrome (WS4) in humans\textsuperscript{42}. A later study confirmed that this highly conserved region contains an important cis-regulatory element for SOX10 expression during melanocyte development\textsuperscript{43}. The homologous element in chicken is studied in paper III.

Eumelanosomes and pheomelanosomes

Melanosomes are lysosome-related organelle structures located within melanocytes (Figure 2). The early melanosome has been suggested to originate from unstructured and round vesicles appearing from the endoplasmic reticulum (ER). This stage I melanosome is a vesicle which evolves to a stage II (eu)melanosome (premelanosome) by developing into a fibrillar, tyrosinase-positive organelle. Synthesis of melanin begins as soon as the fibrillar matrix has been produced, and pigment is deposited on those fibrils (now stage III (eu)melanosome). In highly pigmented cells the deposition of melanin proceeds until the (eu)melanosome is packed and no fibre structure is observable (stage IV (eu)melanosome)\textsuperscript{44, 45}. The round pheomelanin premelanosomes are less structured than the oval eumelanin premelanosomes and contain less melanin\textsuperscript{46} (Figure 2). Melanosomes are used as models for understanding the morphogenesis of lysosome-related organelles and may help in understanding the etiology of disorders linked with lysosome-related organelles\textsuperscript{47}.

Pigmentation in birds and mammals results to a large extent from the deposition and assembly of two different types of melanin, (brown-to-black) eumelanin and (yellow-to-reddish-brown) pheomelanin. The melanin biosynthesis occurs in the melanosomes, and the rate-limiting enzyme of this synthesis is tyrosinase (TYR). Tyrosinase and the tyrosinase-related proteins TYRP1 and TYRP2 (DCT) are involved in the production of eumelanin. For the assembly of pheomelanin, only cysteine (or glutathione) and some tyrosinase activity seem to be critical. When tyrosinase is expressed at low levels, pheomelanin is produced by the addition of cysteine to dopaquinone.
Simplified, high tyrosinase activity results in eumelanin production whereas low activity results in generation of pheomelanin.

Figure 2. A schematic drawing of the development of pheomelanosomes and eumelanosomes within the melanocyte. The round pheomelanin premelanosomes contain less melanin and are less structured than the oval pre-eumelanosomes (stage II and III). In the stage II and III premelanosomes developing into eumelanosomes the PMEL17 protein results in a fibrillar matrix on which the eumelanin pigment can be deposited in an organized fashion until no fibre structure is observable (stage IV). High tyrosinase activity (fat arrow) together with TYR P1 and DCT results in eumelanin and low tyrosinase activity and some cysteine results in pheomelanin. MATP is believed to direct TYR, TYRP1 and DCT from the trans-Golgi-network to stage II premelanosomes. (Figure modified from Hearing 200545)

PMEL17
PMEL17 (pre-melanosomal protein 17) is an integral membrane protein, also known as gp100, SILV and MMP115. Transcription of PMEL17 is regulated by MITF50. PMEL17 is sufficient to drive the formation of the fibrils found in the premelanosome and therefore is an essential component of premelanosome biogenesis51. Critical for the development of these fibrils is the cleavage of PMEL17 by a furin-like proprotein convertase47. PMEL17 polymerizes into fibrillar arrays (the eumelanosome backbone) upon which the melanin pigment is assembled52(Figure 2). These arrays have been found to be the first functional amyloid structures in nature and should be referred to as amyloidin53.

The first mutation found in Pmel17 is causative for a recessive phenotype first described in a black mouse strain that became progressively lighter (more silvered) with age54. The silver (si) mice were found to have a G to A transition that produce a nonsense mutation in the sequence encoding the C-
terminus of the protein, generating a premature stop codon, resulting in truncation of the last 25 amino acids\textsuperscript{55, 56}.

**SLC45A2/MATP**

The membrane associated transporter protein (MATP) is also known as AIM-1 and SLC45A2 and the function of this protein is not yet fully understood. MATP has 12 predicted transmembrane regions and shows sequence and structural similarities to plant sucrose transporters\textsuperscript{57}. Transcription of \textit{MATP} is also regulated by the MITF transcription factor and it is believed to play a crucial role in directing tyrosinase and TYRP1 (and DCT?) from the trans-Golgi network to stage II melanosomes. Mutations in \textit{MATP} is thought to disrupt this traffic, most probably by interrupting the sorting of vesicles between trans-Golgi and the melanosomes\textsuperscript{44, 45, 58}(Figure 2).

Mutations in \textit{MATP} have been identified in many vertebrates. The first mutations were detected in the medaka fish. In most of these mutants both skin and eye defects are seen\textsuperscript{47}. During the same time period, a series of alleles at the \textit{underwhite} locus in mouse (\textit{Uw}\textsubscript{\textit{dbr}} > wild-type > \textit{uw} > \textit{uwd}) were also found to represent mutations in \textit{MATP}.

Mutations were also observed in human patients with oculocutaneous albinism type 4 (OCA4)\textsuperscript{58, 59}. The first human \textit{MATP} mutation was identified in a patient with oculocutaneous albinism (generalized hypopigmentation of skin, hair and eyes). This patient was found to be homozygous for a G to A transition in the splice acceptor sequence of exon 2, resulting in the skipping of this exon and thereby deleting the fourth transmembrane region, thus changing the orientation of the following transmembrane regions\textsuperscript{59}. After this, many more mutations in \textit{MATP} have been found in OCA patients by genetic screenings of patient materials from different ethnic origins\textsuperscript{60, 61}. \textit{MATP} polymorphisms have also been associated with normal human pigment variation\textsuperscript{62}.

**Carotenoid pigment**

More than 1000 naturally occurring variants of carotenoids have been found in plants and other photosynthetic organisms, but none of these can be synthesized by vertebrates and therefore needs to be derived from their foods\textsuperscript{63, 64}. In animals the most important function of carotenoids is their function as vitamin A precursors\textsuperscript{64}. Among mammals, particularly primates and ruminants are the ones accumulating carotenoids. As a consequence they can develop yellow fat with age\textsuperscript{63}. In both cattle and sheep, mutations resulting in yellow fat among the animals represent a major and very costly problem for the farmer, because of a marketing and consumer resistance\textsuperscript{65, 66}. Birds and fish use carotenoids in many external structures, for example the pink colour of flamingos and salmon are due to carotenoids\textsuperscript{64}. Among birds, males with more colourful ornaments have been found to have a better im-
mune status due to the higher levels of circulating carotenoids, they are also more attractive to females. Carotenoids have therefore been said to be an honest indication of an individual’s health status. Whether this is the reason why humans in different regions of the world prefer to eat chickens with yellow skin remains an unanswered question.

**BCDO2/CMO2**

Two different carotenoid-monooxygenases, CMO1 and CMO2, have been identified in vertebrates. CMO1 cleaves β-carotene by centric/symmetric oxidative cleavage at the C15,C15’ double bond. CMO2 (previously known as BCDO2) cleaves β-carotene and lycopene (a carotenoid pigment, for instance found in tomatoes) by an excentric/assymetric cleavage at the C9’,C10’ double bond. The cleavage of β-carotene is the key step in the formation of vitamin A (retinol), CMO1 has been identified as the key enzyme in this process, and the physiological role of CMO2 is less understood. In vertebrates vitamin A has multiple functions during development and cell differentiation. Retinal and related compounds serve as the chromophores of rhodopsins (visual pigments) in animals. Tissue expression of both CMO1 and CMO2 has been found to be ubiquitous, and vitamin A dependent processes may be tissue specifically regulated thanks to circulating carotenoids. CMO1−/− mice accumulate large quantities of β-carotene in several tissues and have decreased vitamin A levels, this result makes the contribution from CMO2 for vitamin A production questionable. The transcription of CMO1 has been shown to be regulated by PPARs and RXRs, which indicates a regulatory link between carotenoid and fatty acid metabolism, and CMO1−/− mice also gain more weight than wt control animals. Lycopene has been shown to decrease the expression of CMO1 and PPARγ (and to a small extent CMO2) in rats, implying a role for this CMO2 cleaved carotenoid in the modulation of β-carotene and lipid metabolism.
Present investigations

Aims of this thesis
The objectives of this study have been:

- to identify the genes for four major pigmentation phenotypes in chicken (the I, S, Db and W loci), and thereby understand more about the genetics underlying pigmentation variation.

- to explore the chicken domestication process.

Background

Pedigrees and animals

The red junglefowl x White Leghorn (SLU13) pedigree
In 1998, a pedigree for gene mapping between one red junglefowl (RJF) male and three White Leghorn (WL) females of the SLU13 line was generated\(^{27, 73}\). The red junglefowl and White Leghorns are fixed for different alleles at many loci controlling phenotypic traits and the genetic markers used for linkage studies in this cross were tested for informativeness in the parental generation. The cross was initiated by crossing the parental animals, generating F\(_1\) animals. These animals were intercrossed and about 850 F\(_2\) individuals were generated. The initial linkage analysis was performed using \(\sim\)100 evenly spaced markers\(^{73}\). After the initial scan, additional markers were added in the regions of interest. To handle all the data and to perform linkage analysis the CRIMAP software has been used\(^{74}\). A second set of about 350 informative SNPs has also been genotyped in the pedigree. Despite this, many microchromosomes are still not covered. Digital pictures of 814 F\(_2\) animals were used for phenotypic classification\(^{75}\). This pedigree was used in papers I and II.

The red junglefowl x White Leghorn (OS) pedigree
In 2005, an intercross between a second White Leghorn line, the Obese strain (OS) chicken\(^{76}\), and the red junglefowl (RJF) was generated. The Obese strain is a unique model for spontaneous autoimmune thyroiditis, and
this cross was generated to perform genetic analysis of this autoimmune disorder. The cross was initiated by crossing two RJF males with eight OS females and one OS male with two RJF females. From the F1 generation eight males and 35 females were selected to generate the F2 generation of about 800 individuals (Sahlqvist A-S et al. in prep). 356 informative SNPs and some extra markers in interesting regions have been analyzed in the cross using the CRIMAP74 software (Sahlqvist A-S et al. in prep). Also in this cross markers on many of the microchromosomes are missing. All but one batch of the F2 animals got Levaxin (thyroxin) supplemented to their food due to the hypothyroidism in the OS parental line. Digital pictures of high quality were taken of all the F2 birds every third week of their life (at 1, 3, 6, 9, 12, 15, 18, 21, 24 and 28 weeks of age). All pictures were sorted by individual in Extensis Portfolio 6 (Extensis, Inc, Portland, OR, USA) and used for phenotypic classification of the birds. This pedigree was used in paper III. Samples from the RJF and OS line were also used in papers II and IV.

**Samples from international collaborators**

From collaborators at the INRA GFA experimental unit in France, we have received both DNA samples and tissues for RNA extraction. Many of the DNA samples from different breeds were collected by the AvianDiv project77. Samples received from INRA have been used in papers II, III and IV.

From collaborators at Nutreco/Hendrix Genetics in The Netherlands, we received two experimental crosses segregating for the **Silver** and **yellow skin** phenotypes, studied in papers II and IV respectively.

**Loci under investigation**

**The I locus in chicken (paper I)**

The **Dominant white** (I) colour is typical for the White Leghorn breed and it was one of the first traits examined after the rediscovery of Mendel’s laws of inheritance5. This incompletely dominant allele drastically reduces the eumelanin pigment9. The melanosomes of **Dominant white** chickens have also been found to have an irregular shape and assembly78. The I locus was previously mapped to chicken linkage group E22C19W28 which shows conserved synteny with mouse chromosome 10 and human chromosome 1279, 80.

**Dun** (I^D_) is another allele at the I locus. The Dun allele was identified in a Pit-Gamecock bird and gives a brownish colour in heterozygotes and a whitish colour in homozygotes9. A third allele at this locus is causing the greyish **Smoky** phenotype. **Smoky** is allelic to I and arose as a partial phenotype revertant in a White Leghorn line fixed for **Dominant white** (R. Okimoto, B. Payne and D. Salter, unpublished results).
The S and Al loci in Chicken and Japanese quail (paper II)

In 1912 the sex-linked locus for the Silver (S) plumage colour was identified as an inhibitor of red colour in chicken. S is allelic to the recessive red/brown colour (gold, $s^+$) found in the red junglefowl. The mode of inheritance of the Silver phenotype is complex because the phenotype seems to be strongly influenced by modifying genes.

A third allele at the S locus is sex-linked imperfect albinism ($s^{al}$). This is the bottom recessive allele at this locus. The $s^{al}$ birds have a white plumage colour with a ghost patterning that is dependent on genetic background. The eyes are red at hatch, but except for the red pupils they darken with age. In his book from 1949, Hutt stated that sex-linked albinism has also been found in many other domestic bird species, such as turkey, budgerigar and canary. The possibility of studying this phenotype as part of a comparative approach made us interested in this phenotype in Japanese quail (Coturnix japonica).

Imperfect albinism (al) has been identified in at least four populations of Japanese quail. One of the first to report this phenotype was Lauber in 1964. The chicks have bright pink eyes and yellow to white plumage colour and adult birds have white plumage with buff ghost-barring, lacking melanin granules. Melanoblast cells from albino quail differentiate to functional melanocytes containing melanosomes, but without melanin pigment or tyrosinase activity in these melanosomes. However, the albino quail melanocytes still have tyrosinase activity in the Golgi-endoplasmic reticulum-lysosome and in the Golgi vesicles. These results indicate that the al mutation affects the tyrosinase transport from the Golgi to the melanosomes. The imperfect albino phenotype (al) is recessive to wild-type (Al$^+$). By crossing male chickens homozygous for S, $s^+$ or $s^{al}$ to female Japanese quail hemizygous for Al$^+$ or al it was found that the S and Al loci in chicken and quail are orthologous. All offspring generated from the interspecies cross between albinos were albino.

The cinnamon phenotype ($al^C$) is caused by a second mutant allele at the Al locus in quail and considered to be indistinguishable from the dark-eyed dilute ($al^D$) phenotype that has also been found in Japanese quail and been linked to the Al locus. The eyes of the chicks are red and have subnormal melanin pigmentation but darkens with age, and the brown pigments of the feathers are diluted without affecting the wild-type plumage pattern (all phenotypic measurements were described for the $al^D$ phenotype). The $al^C$ allele is recessive to wild-type (Al) but dominant to imperfect albinism (al).

The chicken Db locus (paper III)

In 1972, the Dark Brown (Db) phenotype was described in autosomally barred Fayoumi chickens. In females the pheomelanin appears as an orange-tan or burnt orange colour and males have completely red-brown plumage.
breasts\textsuperscript{89, 90}. The \( Db \) locus is linked to the \textit{autosomal barring} (\( Ab/Pg \)) locus. In a map of chicken chromosome 1, Bitgood and Somes 1990 mapped \( Db \) in between \( P \) (pea comb) and \( Pg \) (pattern/(lacing/autosomal barring/pencilling)), \( P - 33 \text{ cM} - Db - 20 \text{ cM} - Pg \). Previous studies have shown that the expression of the \( Db \) allele is modified by alleles at the \textit{Extension} (\( E \)) locus\textsuperscript{89-92}. The \( E \) locus encodes the melanocortin 1-receptor (MC1R) and affects the relative distribution of the eumelanin and pheomelanin pigments\textsuperscript{75}. The breasts of females with the wild-type allele at \( MC1R (e^+) \) show a salmon-brown colour and the \( Db \) allele has previously been found to have limited effect on these salmon-coloured breast feathers\textsuperscript{9, 90}. \( Db \) has on some genetic backgrounds been found to act as a sex-influenced phenotype, expressed as dominant in males and recessive in females\textsuperscript{91}.

\textbf{The \( W \) locus in chicken (paper IV)}

The \( W \) locus in chicken was first described by Bateson in 1902\textsuperscript{5}. The \textit{yellow skin} (\( W^*Y \)) allele has been considered to be the mutant form because the red junglefowl carry the \textit{white skin} (\( W^*W \)) allele. The phenotype cannot be easily scored until the chicks are 10-12 weeks of age since the \( W \) locus controls the amount of xanthophylls in the skin and is dependent on its deposition. Food high in carotenoids (such as yellow corn) enhances the yellow pigmentation of the skin. During lay, carotenoids are deposited in the egg-yolk, resulting in less pigmented skin of the females during periods of extensive egg laying\textsuperscript{9}. In 1949 Hutt launched the idea that the \textit{yellow skin} phenotype should have been inherited from the grey junglefowl, suggesting a polyphyletic origin of the domestic chicken\textsuperscript{19}.

\textbf{Results and Discussion}

\textbf{Paper I}

\textbf{The Dominant white, Dun and Smoky Color Variants in Chicken Are Associated With Insertion/Deletion Polymorphisms in the \textit{PMEL17} Gene}

In this study, linkage mapping was carried out in the red junglefowl x White Leghorn SLU13 pedigree to confirm the mapping of the \textit{Dominant white} (\( I \)) locus to linkage group E22C19W28\textsuperscript{79}. The segregation at the \( I \) locus did not deviate significantly from the expected 3:1 ratio in the F\textsubscript{2} individuals from the intercross. A sexual dimorphism in plumage colour was noted, this is due to the sexual dimorphism in chicken phenotype and the sex-linked \textit{Barred} and \textit{Silver} loci also segregating in this cross. The candidate gene \textit{PMEL17} was found by comparative mapping with human (chr12) and mouse (chr10) which show conserved synteny with E22C19W28\textsuperscript{80}. A recombination frac-
tion of zero and a LOD score of 107.2 between I and PMEL17, strongly indicated that PMEL17 could be the gene causing the different phenotypes found at the I locus in chicken.

Apart from the Dominant white colour that is characteristic of White Leghorns, the Dun (I$^D$) and Smoky (I$^S$) phenotypes were also examined in this study. The complete PMEL17 gene was sequenced beginning 32 bp before the start codon and including 111 bp of the 3' UTR. A total of 56 SNPs and eight insertion/deletion polymorphisms (indels) were found across populations. Dominant white, Smoky and Dun were all found to be caused by indels disrupting fairly conserved regions in the PMEL17 protein (Figure 3). A 9-bp insertion in exon 10 (723insWAP) was found to be associated with the Dominant white and Smoky phenotypes. The pigmentation defect in Dominant white birds was partially restored in the Smoky birds by a unique 12-bp deletion in exon 6 (280delPTVT), and our interpretation is that this deletion thus reverts part of the protein function. The Dun genotype was clearly distinct from the others with several unique changes, a 15-bp deletion in exon 10 (731delLGTAA) and three amino acid changes (A35V, G105S and R740C) as results of missense mutations. The Dominant white (I) allele was later shown to protect the birds from feather-pecking compared to birds carrying the wild-type (i) allele at this locus

Since this study was performed, mutations have been found in PMEL17 in other species. In dogs, the incompletely dominant merle phenotype (characterized by patches of diluted pigment) has been found to be associated with an insertion of a SINE element at the intron 10/exon 11 boundary in several breeds. Some of these dogs also have auditory and ophthalmologic anomalies. In the zebrafish mutant fading vision (fdv) a point mutation has been found to give a premature stop codon and thereby a truncated protein (lacking the transmembrane domain, the proteolytic cleavage site and the AP binding motif). These mutants have hypopigmentation in skin melanocytes and the retinal pigment epithelium, resulting in a visual defect in the larvae that is restored as the development proceeds. In Silver horses the causative allele specifically reduce the production of eumelanin. Interestingly, the most probable causative mutation results in the same amino acid change (R618C) as one of the missense mutations found in Dun chickens (R740C). The same mutation could also be causative for the Multiple Congenital Ocular Anomalies (MCOA) in horses, but this phenotype might also be due to a mutation in close proximity to PMEL17.

Figure 3. (next page) Alignment of the PMEL17 amino acid sequence associated with the wild type (i) allele present in the red junglefowl, and the Dominant white (I), Smoky (I$^S$) and Dun (I$^D$) alleles in comparison with human (S73003) and mouse (NM_021882) sequences including the mouse silver allele (AF119092). Sequence identities are indicated by dashes and insertion/deletion differences are indicated by dots. The signal sequence, the four copies of the 24-amino acid repeat in chicken, the transmembrane, and the cytoplasmic region are indicated. The arrow indicates the proteolytic cleavage site that generates an aminoterminal Mα and a carboxyter-
Several PMEL17 mutations have been identified in various species, but no complete loss-of-function has been found in vertebrates and the first hu-

minal Mβ fragment. The insertion/deletion polymorphisms associated with I, I° and I° are boxed.

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man mutation is still to be found. PMEL17 is known to be involved in eumelanin formation but the protein may have other functions as well. The fact that no mutation that completely inactivates the function of PMEL17 has yet been discovered suggests that PMEL17 has a crucial but unknown function besides its role in melanogenesis.

Paper II

Mutations in SLC45A2 Cause Plumage Color Variation in Chicken and Japanese Quail

This study demonstrates that mutations in SLC45A2/MATP are associated with the Silver (S), cinnamon (alc) and sex-linked imperfect albinism (s\textsuperscript{al}) and (Al\textsuperscript{+}) phenotypes in chicken and Japanese quail (Figure 4).

By linkage analysis the MATP gene was mapped to the upper part of chicken chromosome Z where the sex-linked Silver locus is known to be located. The nucleotide sequence of all seven exons was determined from genomic DNA of both chicken and Japanese quail. The Silver allele in chicken was found to be associated with a C to A transversion, causing a missense mutation in exon 4 (Leu347Met) in all tested breeds with the Silver phenotype except White Leghorn. This amino acid change is affecting a highly conserved part of the seventh transmembrane region. The S allele found in White Leghorns is associated with an A to G transition, resulting in a missense mutation in exon three (Tyr277Cys) affecting a loop region. This allele was also found in several breeds with the wild-type phenotype. We have been unable to map the Silver phenotype in our RJFxWL(SLU13) pedigree and thus, the phenotypic consequence of this mutation might be highly influenced by the genetic background. Perhaps this mutation shows its effect in pure White Leghorns due to their whole package of pigmentation alleles (I, E, B and S). A one bp deletion (106delT) was found in the chickens with sex-linked imperfect albinism resulting in a frameshift at codon 36 and translation stop in exon one.

In Japanese quail the mutation associated with sex-linked imperfect albinism was found to be a G to T transversion in the last nucleotide of intron three. cDNA sequencing confirmed that exon four is not present in the transcript. The cinnamon allele in Japanese quail is causing a more severe phenotype than the Silver alleles found in chicken, and was found to be due to a C to A transversion in exon one. This results in a nonconservative Ala72Asp amino acid change at a conserved position of the first transmembrane domain.

In summary, the mapping of the contig harbouring MATP to the top of the Z chromosome and the five mutations found, show beyond doubts that mutations in the membrane associated transporter protein (MATP) cause phenotypic variation in chicken and Japanese quail. The incompletely dominant S
phenotype in chicken only inhibits the red (pheomelanin) colour of the plumage. No pheomelanin-specific mutations in \textit{MATP} have been reported earlier. The semidominant D153N mutation found in horse has a stronger effect on pheomelanin production but also affects eumelanin in homozygous form\textsuperscript{97}. Our hypothesis is that one of the functions of \textit{MATP} is to transport the cysteine essential for pheomelanin production into the melanosome, and that this specific function is disrupted in the birds with the \textit{Silver} allele. In the albino birds with loss-of-function mutations all functions of \textit{MATP} is disrupted and neither eumelanin nor pheomelanin pigment is produced.

![Figure 4. Membrane topology prediction of the MATP protein using TMHMM (v. 2.0). The location of the frameshift mutation (S36fs) associated with the \textit{s}\textsuperscript{al} allele and the two missense mutations Y277C and L347M associated with the \textit{Silver} allele in chicken are indicated by black arrowheads. The A72D mutation associated with the cinnamon allele in Japanese quail is marked with a grey arrowhead. The amino acids missing in the MATP protein encoded by the albino allele in Japanese quail is shaded in grey.](image-url)
Paper III

The Plumage Colour Dark Brown in Chicken is Caused by an 8 kb Deletion Upstream of SOX10

In this study we show that an 8 kb deletion located 14 kb upstream of SOX10 results in the Dark brown (Db) phenotype in chicken (Figure 5). The Db phenotype was scored by examining pictures from each of the 765 F2 individuals in the OSxRJF intercross. 161 individuals were scored as having the Db phenotype (41 females, 62 type 1 males and 58 type 2 males). In females the phenotype was seen as an orange tan over the entire plumage except for the tail feathers. In males two different categories of the phenotype was scored. The type 1 males had a bright brown/red/orange breast, but no pheomelanin was seen in the tail, the type 2 males had a similar but a less apparent phenotype. A two-point linkage study including the 41 Db females confirmed linkage of the locus to chicken chromosome 192. No causative mutation was found within the protein encoding parts of SOX10, but when examining conserved regions around the gene the same region as the one found to be deleted in the Hry mice was found to be missing in the Dark brown individuals.

Resequencing of two db+ (RJF) birds and six birds from different lines exhibiting the Db phenotype resulted in a 12.8 kb haplotype (IBD region) shared among all sequenced birds with the Db phenotype, thus excluding all polymorphisms found outside this area as causative mutations (Figure 5). Unexpectedly, one of the sequenced RJF individuals carried a haplotype that was identical to the Db haplotype except for the deletion and its associated 10 bp insertion. This result strongly supports the proposition that the deleted region is causative for the Db phenotype, as the 8 kb deletion is the only unique sequence difference between the Db and db+ chromosomes. In addition to this result the deletion was present in 20 chickens known to carry the Db allele, but in none of the 43 tested birds not expected to carry the Db allele.

Among the type 1 males 49 were found to be homozygous for the deletion and 13 were heterozygous. In the type two group only three were found to be homozygous Db. In this group most individuals (49/58) were instead heterozygous for the deletion, explaining the Db locus as co-dominant, with a more pronounced phenotype in the homozygous individuals. In the type 2 group, six males were also found to be homozygous wt (db+), this result is most probably due to a misclassification when scoring the phenotype. All 41 females phenotyped as Dark brown were found to be homozygous for the deletion. This implies, in agreement with previous studies, that the expression of the phenotype also is influenced by the sex of the bird.

The Hry mouse strain has as previously mentioned a 15.9 kb deletion of a non-coding conserved sequence 47 kb upstream the Sox10 transcription start site. The deleted conserved element is corresponding to the one found in this
study, and it has also been further examined as a segment likely to play a role in Sox10 expression during the migration and development of melanocytes.\textsuperscript{43}

\textit{Db} shows a stronger pheomelanistic expression in the breast (ventral) than in the tail (dorsal) part of the birds. One idea is that SOX10 is differently expressed in breast and tail another hypothesis is that the pattern is a result of a dose relationship between agouti (ASIP) and MC1R. Both these hypothesis are at present under study, as we attempt to confirm that the 8 kb deletion is a cis-acting regulatory mutation.

![Figure 5. The location of an \textasciitilde{}8 kb deletion upstream of SOX10 associated with the Dark brown phenotype (Db_DEL). The borders of the 12.8 kb haplotype showing complete association with the \textit{Db} allele across populations are also indicated (Db_HAP). The figure also shows the coding part of SOX10 and sequence conservation between species. The figure was generated using the chicken genome assembly as presented on the UCSC Genome Browser (www.genome.ucsc.edu, Chicken May 2006 Assembly).]

\textbf{Paper IV}

\textbf{Identification of the Yellow Skin Gene Reveals a Hybrid Origin of the Domestic Chicken}

In this study we show that the \textit{yellow skin} phenotype in chicken is caused by a cis-acting and tissue specific regulatory mutation(s) that inhibit the expression of \textit{BCDO2/CMO2} in skin. We also conclude that there must be a hybrid origin of the domestic chicken, given that the \textit{yellow skin} allele is derived from the grey junglefowl.

The \textit{yellow skin} (\textit{W}) locus had previously been assigned to chicken chromosome 24.\textsuperscript{80} In this study the \textit{W} locus was initially mapped close to the \textit{APOA1} gene (\(\Theta = 0.07\), LOD = 16.4) at the distal end of chromosome 24. This was done by the use of a backcross pedigree (Y/WxY/Y) comprising 91 individuals. Partial sequencing of the candidate gene \textit{BCDO2} resulted in a SNP fixed in all tested breeds with the \textit{yellow skin} phenotype. Further sequencing resulted in a 23.8 kb IBD region where all these breeds show the exact same haplotype (Figure 6).
No obvious causative mutation was found within the coding sequence and thus the effect was supposed to be a regulatory mutation acting on the BCDO2 gene. A pyrosequencing test on cDNA from six heterozygous individuals showed that the BCDO2 expression was clearly affected in skin (90% of the transcripts expressed in skin originated from the white skin allele), but not in liver (Figure 6).

In comparison with chickens carrying the white skin allele (including red junglefowls) a higher sequence divergence (0.81%) than the expected 0.5%\textsuperscript{26}, was seen between the haplotypes at the yellow skin locus. This indicated that the yellow skin allele might in fact be descendant from some of the other junglefowl species as proposed by Hutt\textsuperscript{19}. For the BCDO2 haplotype region the yellow skinned individuals clustered with the grey junglefowl, whereas for all other tested regions of the genome they clustered with the red junglefowl. This implies that the biggest part of the chicken genome is descendant from the red junglefowl, but that small selected parts have been inherited from the grey junglefowl. Whether or not the other two junglefowl species have also contributed to the domestic chicken remains to be shown.

Since this study, research groups in Australia and New Zealand has found a stop codon in BCDO2 to have a large effect on fat colour in cattle\textsuperscript{65}.

Figure 6. (A) Gene content of the yellow skin interval and the 23.8 kb yellow skin IBD region indicated by a box. The annotation is based on the chicken genome assembly (www.genome.ucsc.edu, Chicken May 2006 Assembly). (B) Differential expression of the BCDO2 transcript in skin but not liver from yellow skin heterozygotes using genomic DNA (gDNA) as control. The polymorphic position chr24:6,268,434 bp was used to monitor differential expression using pyrosequencing. T and C at this position correspond to the white and yellow skin alleles respectively. (C) Summary of the examination of differential expression in skin and liver from six heterozygous (W/Y) birds. Genomic DNA from the three different genotypes were used as controls.
Future prospects

In this thesis I have presented data from genetic studies of domestic chickens. During the domestication, humans have selected numerous mutations affecting the phenotype of the birds. The same is true for all of our domestic animals (and plants). By the fast evolving sequencing technologies, sequencing and resequencing of many more species and breeds will be seen in the near future. From these studies we will have the possibility to learn more about the evolution of different species, but also more about genome structures, the functional elements in the genomes, and be able to pin-point selective sweeps and mutations underlying phenotypic diversity. These sequencing efforts will also give us an almost unlimited number of genetic markers to test in breeds and pedigrees for the fine-mapping of trait loci. The possibility of sequence capture technology, to select a candidate region of up to a Mb for resequencing, will tremendously speed up the process of finding the causative mutation.

No $PMEL17$ mutation has yet been found in humans and there is not yet any knockout mouse for this gene. If $PMEL17$ is only affecting pigmentation a “spontaneous” knockout would have been expected to be found and studied by now, given the extensive screening for coat colour mutations in the mouse. The fact that a knockout has not been seen in any species suggests that $PMEL17$ has other functions that are essential for survival, and that a complete loss-of-function mutation is lethal. Since $PMEL17$ primarily affect the assembly of eumelanin, a study of red-haired-humans lacking eumelanin pigmentation could reveal the first human mutations in this gene.

Given that no pheomelanin-specific mutations in $MATP/SLC45A2$ have been reported earlier it would be of interest to learn more about the function of $MATP$, especially its respective effect on pheomelanin and eumelanin production. Why do some mutations in this gene affect the synthesis of both eumelanin and pheomelanin, whereas other inhibits the production of pheomelanin but leaves the production of eumelanin unaffected?

Hutt claimed that the Silver allele in chicken may have been derived from the grey junglefowl. By sequencing exon 3 and 4 of the $MATP$ gene in Grey junglefowl we can exclude this statement, since the grey junglefowl did not have any of the two Silver mutations found in paper II (unpublished results). The grey phenotype in this species might still be a result of a $MATP$ mutation and sequencing the rest of the exons could perhaps answer this. Since the turkey, budgerigar and canary are suspected to have sex-linked
albinism caused by mutations in the same gene as chicken and Japanese quail, *MATP* is an obvious candidate gene for these phenotypes as well. With respect to the yellow skin locus (paper IV), Hutt was correct about the phenotype deriving from the grey junglefowl. By whole genome resequencing of the three additional junglefowl species (the Ceylon, grey and green junglefowl) we could learn if more of the grey junglefowl genome has been selected for in our domestic breeds and then perhaps find some more causative mutations for traits deriving from this species. If the Ceylon and green junglefowl have somehow contributed to the domestic chicken is currently unknown and resequencing of these genomes could therefore give us more knowledge about the domestication process.

A knockout mouse has been created for the *CMO1* gene, resulting in an accumulation of β-carotene and prevention of vitamin A production. This knockout also suffered from damaged lipid homeostasis. No knockout mouse has been generated for the *CMO2/BCDO2* gene and the function of this enzyme has been questioned. At a recent conference a stop codon in *CMO2* was described in cattle. I do not know where in the gene this stop codon is located or if there are more phenotypes in these animals than the yellow fat. Until this has been carefully studied I think it could be of interest to knock out the *CMO2* gene in mice to determine how it resembles/differs from the phenotype of the *CMO1* mice. Perhaps it could also be interesting to generate *CMO1/CMO2* double knockouts to investigate whether these enzymes have overlapping functions that are totally disrupted and results in a more severe phenotype if both genes are inactivated. It would also be of interest to understand which of the polymorphic sites within the 23.8 kb haplotype region for yellow skin that is causative for the phenotype and what factor that binds there. This would improve our knowledge about the regulation of this gene.

Expression studies of the Dark brown allele (paper III) should be performed. How does this deletion affect the eumelanin distribution and give an expression of pheomelanin instead? How is this deletion different from that of the *Hry* mouse (which has a more severe phenotype, affecting both melanocyte migration and resulting in megacolon)?
Genetiska studier av hönsens färgteckning

I min avhandling har jag studerat gener som reglerar pigmentering. Färgfenotyper är enkla att avläsa visuellt för varje enskild individ och följer ofta en enkel monogen nedärvning. Detta gör att generna som styr pigmentering är värdefulla modeller för att förstå hur gener fungerar och integrerar med andra gener. Jag har under min doktorandtid till stor del studerat dessa fenotyper i två unika korsningar mellan den röda djungelhönan (Gallus gallus) och två olika linjer av vit Leghorn.

I den första artikeln studerades det lokus (kromosomregion) som ger dominant vit färg hos höns. Här fann vi koppling mellan den vita färgen och PMEL17 genen. Efter karakterisering av genen hos höns med olika fjäderfärger fann vi tre mutationer som ger tre olika fenotyper (egenskaper, i detta fallet fjäderfärger). Två av mutationerna (dominant vit färg och Dun mutatie) hindrar uttrycket av eumelanin (svart pigment) och orsakas av en insertion på tre aminosyror respektive en deletion på fem aminosyror i transmembranregionen av PMEL17. Smoky mutationen uppstod som en partiell reversionsupptäckt inom en linje som är homozygot för mutationen som ger dominant vit färg och har därför både insertionen på tre aminosyror i transmembranregionen, samt en deletion av fyra aminosyror i den del av proteint som kodas av exon sex.

leder till att hela exon fyra klyvs ut med resultatet att proteinets struktur drastiskt förändras.

I den tredje artikeln fann vi koppling mellan fenotypen Dark brown (Db) och SOX10 genen hos höns. Denna fenotyp nedärvs dominant i tupparna och recessivt i hönorna i en korsning mellan röd djungelhöna och vit Leghorn. Vi visar att ett ~8 kb stort fragment av ett konserverat och tidigare studerat regulatoriskt element för reglering av pigmentceller saknas hos alla individer med Db fenotypen. Alla andra möjliga polymorfrier mellan Db individernas haplotyp och vildtypen kunde uteslutas.

I det fjärde och sista delarbetet studerades det lokus som ger upphov till gul hud hos många domesticerade hönsraser. Denna fenotyp är orsakad av en recessivallel (W*Y) som tillåter deponering av gula karotenoider i huden. I detta arbete visar vi att den gula färgen orsakas av en vävnadsspecifik regulatorisk mutation som inhiberar uttrycket av BCDO2 genen i huden. I höns av vildtyp (W*W) som saknar mutationen bryter BCDO2 ner karotenoider som tagits upp genom födan, men denna funktion har gått förlorad i individer med gul hud. En annan mycket spännande upptäckt under detta arbete var att allelen för gul hud härrör från den grå djungelhönan (Gallus sonneratii) och därmed kullkastades den tidigare allmänna uppfattningen att den röda djungelhönan var den enda ursprungsarten till dagens tamhöns.

Arbete tre och fyra visar på betydelsen av regulatoriska mutationer som orsak för fenotypisk diversitet.
Acknowledgements

I would like to express my gratitude to ALL people that I have spent time and collaborated with during my PhD studies. You have made this thesis possible!

Especially I would like to thank:

Min huvudhandledare Leif Andersson, tack för att du med ditt lugn och din passion för forskningen väglett mig och ökat min förståelse för vetenskapliga frågeställningar. För mig har dessa år varit väldigt lärorika och något av en känslomässig berg-och-dalbana i vissa perioder. Tack för att du tagit dig tid när jag behövt det. Lycka till med alla projekt i framtiden! 😊


Min biträdande handledare Göran Andersson, tack för kommentarer på min avhandling och andra texter under dessa år. Jag uppskattar också ditt intresse för mina projekt och allmän uppmuntran under min doktorandtid!

Frida, Lina, Ellen & Sus! Jag tycker att människorna är minst lika viktiga på en arbetsplats som arbetsuppgifterna och ni har verkligen bidragit till en trevlig arbetsmiljö! 😊 Kul att vi fortsätter att träffas, snacka och åta då och då nu när vi börjar spridas för vinden.

Per, Anders & Jonas, det har varit kul att jobba med er! Ni har alla mycket spännande på gång, lycka till med allt pojkar (eller män menar jag såklart, sorry)!

Min rumskompis Freyja, det har varit roligt och intressant att dela rum med dig!

Ulla, du är en klippa! 😊 Tack för allt du lärt mig under åren och för fint samarbete med alla pyrokörningar!
Gudrun, jag saknar dig! Du har varit ett enormt stöd. Hoppas du trivs med livet som pensionär och farmor. Tur att det är många disputationer nu i vår så att man får chans att träffa dig lite!

Göran H & Erik B-R, tack för allt ni hjälp till och fixat med!

Alf, det var underbart att jobba med dig! Du fixade allt och var jättetrevlig när man kom och hälsade på dig och hönsen i Skara!

Stina, alla bilder du tog på hönsen har varit guld värd!

Jen, thanks for helping out with the pictures!

I would also like to take this opportunity to thank past and present members of the ”chicken group”. It has been interesting to discuss science with you at our Tuesday meetings, and I enjoyed getting to know some of you a bit better during the trips to Skara! I would also like to thank all our Swedish and international collaborators for good team-work during these years. I hope it will continue!

And again, all past, present and new members of the lab! You have all contributed to the atmosphere and I will really miss Fredagsfika! Hopefully I can sneak in now and then during the autumn as well to meet you all! Will this Café ever become a reality, as a spin-off from our labs love for cake?

E-spik för all hjälp med avhandlingens utformning.

Gijs Afinx, thanks for introducing me to the world of science during my ”forskningspraktik”! You were a really good teacher and I still perform many procedures in the lab exactly the way you taught me. 😊

Alla personer som jag lärt känna och fick jobba med under UGSBR-året! Det var jätteskoj, skulle gärna köra en runda till! Också stort tack till alla som var med och gjorde SNiB-konferensen i Uppsala möjlig. Många trevliga middagar blev det!

Julia, Erika, Therese & Veronica ni gjorde grundutbildningen till en dans på rosor! Jag har haft så mycket skoj med er. Hoppas jag ska bli lite bättre på att hålla kontakten när den här hektiska perioden är över.

Jonas, Eva, Linus & Malin, stort tack för att ni tagit hand om min familj på bästa sätt under alla helger som jag häckat på BMC. De har haft jätteroligt varje gång och jag har varit så avundsjuk… Hoppas vi kan hitta på fler roliga saker i vår!
Katarina, Thomas & David, tack för mycket trevligt resesällskap! Jag hoppas på en ny resa 2010 eller så, kan det passa? Skulle vara superkul!

Eva N, det är skönt att alltid ha någon att ringa när man behöver prata av sig lite. Hoppas vi ses snart!

Britta med familj, jag hoppas på många trevliga lekstunder i vår/sommar!

Alla trevliga barnfamiljer, förskolefröknar & grannar vi lärt känna de senaste åren, känns jättekul att bli lite riktig Uppsalabo efter sisådär 10 år i stan. Nu vill jag stanna här hos er för ni är så himla trevliga!

Släkt & vänner i norr (och på andra sidan jordklotet) samt den ingifta släkten i söder! Det är superskönt att få komma och hänga med er under sommarsemester och julledighet. Tack för att ni finns!

Mian, Christophe, Hugo, Alex & Edwin! Längtar till fjällresan i påsk, ska bli så kul att träffa er alla!

Birgitta & Anna, vad skulle vi göra utan er? I höst måste vi nog komma och plocka några hundra liter lingon, så mycket Anna-sylt som vi gör av med. Birgitta du ska ha stort tack för att du alltid ställer upp med stort som smått!

Mamma, Pappa & småbrorsorna Anders & Stig, tack för att ni peppt och trott på mig! Ska bli mycket spännande att se vad som händer när det här är klart… kan inte svara på det just nu, men först blir det lite paus med utökning av släkten i alla fall!

Martin & Vilgot, mina grabbar, ni är verkligen toppen! 😊 Vad skulle jag göra utan er? Nu när det här är färdigt hoppas jag att vi ska få mycket mer tid att mysa och hitta på roliga saker tillsammans! Jag älskar er ♥.
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