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# Genomics of chicken domestication and feralisation

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# Abstract

Domestication can serve as a study system of rapid evolutionary change with wide-ranging effects on traits in animals. The chicken was domesticated from the Red Junglefowl and has diverged in behaviour, morphology and life history traits. Conversely, feralisation is a more recent process when domestic animals are again exposed and respond to an environment outside of human husbandry. Linkage-based quantitative trait locus (QTL) mapping has been used to localise genetic variants that affect domestication traits in the chicken genome. Because of the limited resolution of linkage mapping, the QTL regions associated with domestication traits are often broad and contain many genes. One approach to help sort out potential causative genes is to measure gene expression as an intermediary molecular phenotype. In this dissertation, expression quantitative trait locus (eQTL) mapping of gene expression traits is used to search for potential causative genes for domestication traits in the chicken. Expression quantitative trait loci were mapped across the whole genome in bone and hypothalamus samples, and targeted at QTL regions in the base of the comb. These studies have resulted in candidate quantitative trait genes, supported by genetic and gene expression evidence, for relative comb mass, bone allocation, egg production and fearful behaviour as measured in an open field test. Secondly, a population genomics approach was used to study the molecular basis of feralisation in a free-range feral chicken population from the Pacific island of Kauai. Mitochondrial DNA sequences and phenotypic observations establish the hybrid origin of this population as a mixture of wild and domestic chickens. Genome-wide mapping of pooled heterozygosity highlight regions that may be involved in adaptation to the feral environment. The expression QTL results bring us closer to knowledge about the molecular basis of domestication traits in the chicken, suggesting plausible candidate genes and opening up for functional studies of individual loci. The population genomic study shows that feralisation has a mostly different genomic architecture than domestication, and suggests phenotypic effects, based on overlap with domestication QTL regions, for some of the identified regions.

# Populärvetenskaplig sammanfattning

Domesticering är när en vild population ger upphov till en tam population i människans vård, och är ett exempel på snabb evolutionär förändring som påverkar många ärftliga egenskaper hos djur. Feralisering, å andra sidan, är när tama djur åter utsätts för vilda omgivningar. De stora ärftliga skillnader som uppstått under domesticering gör att jämförelsen mellan vilda och tama djur kan användas som genetiskt studiesystem för flera kvantitativa egenskaper. Hos feraliserade djur kan man se vad som händer med tamdjurs egenskaper och genetiska varianter i en vild miljö.

Den här avhandlingen handlar om den genetiska grunden för domesticering och feralisering. Den omfattar arbeten som belyser, i tur och ordning: hönskammens storlek; benets tjocklek och densitet; äggproduktion; rädsla i form av beteende i ett open field-test; de frigående hönsens ursprung på Kauai; samt gener som visar spår av naturligt urval hos hönsen på Kauai. Projekten har haft två övergripande syften: att använda genetisk kartläggning av genuttryck för att närma sig de orsakande generna bakom domesticeringsegenskaper, samt att beskriva feralisering på Kauai med hjälp av DNA-sekvenser.

Idén är att kombinera genetisk kartläggning av kvantitativa egenskaper (på engelska *quantitative trait locus mapping*, QTL) med genetisk kartläggning av genuttryck (*expression quantitative trait locus mapping*, eQTL) för att hitta gener som förklarar ärftliga skillnader i domesticeringsegenskaper. Genetisk kartläggning är en familj av statistiska metoder som går ut på att koppla individers egenskaper till genetiska varianter. Man mäter egenskaper hos besläktade individer och tar reda på deras genotyper på en mängd kända varianter, som fungerar som genetisk karta, och hittar varianter som är associerade med egenskapen. På så sätt pekar genetisk kartläggning ut delar av genomet, QTL-regioner, som innehåller orsakande varianter. När en genetisk variant agerar genom att påverka uttrycket av en gen, vilken i sin tur påverkar en egenskap, så kommer samma regioner att vara associerade både med egenskapen och med uttryck av genen ifråga. Därför går det att lokalisera sådana gener genom att kombinera QTL-regioner från kartläggning av egenskaper och kartläggning av genuttryck.

Hönskammen är ett sexuellt ornament, det vill säga en egenskap som höns finner attraktiv hos en partner. Kamstorleken har också ökat med domesticering, så att tamhöns har större kam än vilda höns även i förhållande till sin större kroppsvikt. Det finns flera möjliga mekanismer som kan förklara hur sexuella ornament kan uppstå och finnas kvar i en population, och många av dem förutsätter att det finns genetiska varianter som har flera effekter (så kallad pleiotropi) på både ornamentet och andra egenskaper. Mycket riktigt är kamstorlek genetiskt kopplat till äggproduktion och investering i ben. Här kombineras genetisk kartläggning av kamstorlek med genuttrycksmätningar från vävnaden vid kammens bas.

Hos fåglar finns ett samband mellan omsättningen av material i ben och äggproduktion. Honor, däribland hönor, producerar en särskild form av medullärt ben som tjänar som kalciumreserv för äggskal. Det betyder en potential för pleiotropi mellan genetiska varianter som påverkar ben och ägg, samt att hönor som lägger många ägg riskerar att bli bensköra. Här analyseras röntgenmätningar och genuttryck från lårben på samma sätt som med kammen, men i större skala och med genuttrycksmätningar från hela genomet. Dessutom används genuttrycksdata från ben och från hypothalamus, som är inblandad i neuroendokrin reglering av bland annat fortplantning, för att söka efter gener som är inblandade i äggproduktion.

Den kanske mest uppenbara skillnaden mellan tama och vilda höns, förutom utseende, är att tamhöns är mer lättskrämda. Det är också en skillnad som måste vara viktig för domesticeringen: höns som är mindre rädda för människor kommer antagligen klara sig mycket bättre i en miljö som styrs av människor, medan det omvända sannolikt gäller i en vild miljö. Ett sätt att mäta rädslan är att sätta en fågel i en öppen arena och mäta hur den rör sig. Sådana mätningar kombineras med genuttryck från hypothalamus, för att hitta gener som är inblandade i regleringen av rädsla. I förlängningen kan sådan kunskap vara användbar för att bättre förstå mekanismerna bakom rädsla och ångest i andra djur, inklusive människor.

Feralisering är, på ett ytligt plan, omvändningen av domesticering. Sett till vilka delar av genomet som påverkas av naturligt urval, och alltså vilka gener som är inblandade, verkar det däremot vara en annorlunda process. För att studera feralisering DNA-sekvenserades hela genom från frilevande höns på Kauai. Dessa höns sägs härstamma både från vilda höns och förrymda tamhöns som feraliserats, och studier av Kauai-hönsens fjäderdräkt, galanden och mitokondrie-DNA bekräftar den bilden. Vidare identifierades delar av genomet som har reducerad genetisk diversitet på Kauai, vilket tyder på att de innehåller gener som nyligen upplevt naturligt urval, och alltså är viktiga för anpassning. Av de regioner av genomet som har låg diversitet är de flesta oberoende av de som har låg diversitet i domesticerade höns, vilket tyder på att mestadels olika gener är inblandade i feralisering och domesticering.

Sammantaget har dessa studier funnit ett antal potentiella kandidatgener för kamstorlek, investering i ben och äggproduktion, rädsla, och slutligen feralisering. Många av dessa gener är okända eller litet studerade i samband med dessa egenskaper. Detta öppnar för molekylärgenetiska studier som kan hitta genetiska mekanismer, och populationsgenetiska studier som kan beskriva hur orsakande varianter sprids i populationer.

## Papers

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- III. Johnsson M, Jonsson KB, Andersson L, Jensen P, Wright D. (2015) Genetic regulation of bone metabolism in the chicken: Similarities and differences to mammalian systems. *PLOS Genetics* 11 (5) e1005250
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- V. Johnsson M, Jonsson KB, Andersson L, Jensen P, Wright D. Quantitative trait locus and genetical genomics analysis identifies putatively causal genes for fecundity and brooding behaviour in the chicken. *Submitted manuscript*.
- VI. Gering E, Johnsson M, Willis P, Getty T, Wright D. (2015) Mixed ancestry and admixture in Kauai's feral chickens: invasion of domestic genes into ancient Red Junglefowl reservoirs. *Molecular Ecology* 24 (9) 2112–2124
- VII. Johnsson M, Gering E, Willis P, Lopez S, Van Dorp L, Hellenthal G, Henriksen R, Friberg U, Wright D. The genomic signals of feralisation: Not just domestication in reverse? *Submitted manuscript*.

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## Papers not included in thesis

Nätt D, Rubin C-J, Wright D, Johnsson M, Beltéky J, Andersson L, Jensen P. (2012) Heritable genome-wide variation of gene expression and promoter methylation between wild and domesticated chickens. *BMC Genomics* 13 (59)

Persson ME, Roth LSV, Johnsson M, Wright D, Jensen P. (2015) Human-directed social behaviour in dogs shows significant heritability. *Genes, Brain and Behavior* 14 (4) 337–344

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# Background

## Introduction

The chicken was domesticated around 6 000 to 10 000 years ago in Southeast Asia from the Red Junglefowl (Tixier-Boichard, Bed'hom et al. 2011; Xiang, Gao et al. 2014), and has probably also seen some introgression from the Grey Junglefowl (Eriksson, Larson et al. 2008). Domestication, here understood as the evolutionary process when a wild population changes in response to a life with humans, clearly has multiple causes (Price 1984; Zeder 2012; Larson and Fuller 2014). It involves natural selection in coevolution of the human and domestic species, as well as relaxation of natural selection pressures such as a reduction in predation. Neutral processes such as population bottlenecks and founder effects must contribute as well. Particularly during later breed improvement, there is intense artificial selection as well as the abolishment of sexual selection when humans control of reproduction.

One may study the genetics of domestication for various reasons. Firstly, one may use domestication as a study system for traits that have diverged during domestication and of rapid phenotypic evolution. Secondly, one may be interested in improving the productivity or welfare of domestic animals through breeding, and search for useful genetic variants that can be incorporated into breeding programs. Thirdly, one may be interested in the particular problem of correlated selection responses in domestication. The research reported in this dissertation mainly takes this first perspective, exploiting the large divergence of domestic White Leghorn chickens from the Red Junglefowl to map the genetic basis of domestication traits. It uses gene expression measurements and studies of a feral chicken population to study the genomic architecture of domestication.

Genetics and genomics deal with the inheritance of traits through genes and genetic variants. The word “gene” is not necessarily easy to define, but in modern use, it usually refers to a DNA sequences that has some function (Griffiths and Stotz 2013). Generally, it is transcribed to a protein-coding messenger RNA or another functional RNA. Part of genetics is to study the normal function of genes in cells and organisms. Another part of genetics, to which this dissertation belongs, aims to explain heritable differences between individuals. Such differences are caused by genetic variants, or alleles. A causal variant affects the function of a gene in such a way that individuals who carry one variant (allele) of the gene differ in some organismal phenotype from individuals that carry another variant of the same gene. One aim of genetics is to isolate and study causal variants. However, sometimes it is easier to find the genes whose functions have been altered by variants, before the sequence variants themselves. Throughout the dissertation, such genes will be referred to as “quantitative trait genes” and “causal genes” when they mediate the effect of a genetic variant on a quantitative trait. It should

not be taken to imply that the only, or even most important, function of that gene is in the trait in question, or that the causal variant is necessarily in that protein-coding gene. In fact, most of the time, the discussion will be restricted to variants that affect the regulation of gene expression, rather than change protein-coding sequences.

Causative variants bring about their effects through some perturbation of molecular and physiological processes inside the organism, and fundamentally inside a cell. Any downstream effect on organismal phenotypes will be mediated by intermediary molecular traits. Such traits could include enzyme activities, concentrations of gene products and secondary messengers, metabolites, secreted hormones, cellular proliferation and migration, and so on, including any aspect of cellular biology and physiology that can be measured. The ability to simultaneously measure the transcript abundance of every known gene in a tissue sample opens for the possibility of using gene expression as an intermediary molecular trait to highlight genes involved in a trait.

Though any given gene is often named after any first crumb of functional information that was discovered about it, genes often participate in multiple biological processes. This notion makes intuitive sense, but is not necessarily easy to delineate. For the purpose of this dissertation, a pleiotropic variant is one that affects more than one trait, where the traits can be regarded as sufficiently different as to not be redundant measures. Rockman and Paaby identified at least three perspectives on pleiotropy; the above is what they term “developmental pleiotropy” (Paaby and Rockman 2013). It is developmental in the sense that the genetic variant changes something about the process of ontogeny that has multiple outcomes. There are also attempts to create taxonomies of different ways that developmental pleiotropy may come about: by a single variant that affects multiple otherwise functionally unrelated genes, by downstream effects on multiple pathways, metabolite levels and so on (Hodgkin 1998). However, such a detailed level of knowledge about gene action does not exist for the genes discussed in this thesis. Hopefully, future studies may reveal such information for some of the loci detected here.

With these definitions out of the way, we may proceed to discuss the domestication traits considered and the methods employed to study them.

## The comb as a sexual ornament

A sexual ornament is a trait of some kind, be it morphological or behavioural, which attracts the opposite sex. The ornamental trait may be an honest signal of mate quality, but once a heritable preference is established in a population, entirely arbitrary traits may evolve in so called Fisherian runaway selection (Mead and Arnold 2004). The chicken comb is sexual ornament (Zuk, Thornhill et al. 1990; Pizzari and Birkhead 2000; Pizzari, Cornwallis et al. 2003; Cornwallis and Birkhead 2007) in both Red

Junglefowl and domestic chickens. Comb size has also increased in domestication, resulting in domestic chickens displaying larger combs, even relative to their greater body mass. When one sex invests more resources into the offspring, there is bigger scope for competition among the other sex, which explains why sexual ornaments are often associated with males. However, in the chicken, comb size appears to play an ornamental role both in males and females. Also, several variants that affect relative comb mass appear to have pleiotropic effects on, or be linked with variants that affect bone parameters and egg production (Wright, Kerje et al. 2008; paper V), age at sexual maturity (Wright, Rubin et al. 2012), as well as spur and testis size (Von Schantz, Tufvesson et al. 1995). These connections between comb mass and other traits that may affect fitness do not rule out other mechanisms, such as Fisherian trait exaggeration, but they mean that to some extent, relative comb mass reflects mate quality. These functional connections also point to potential trade-offs between natural and sexual selection that may constrain comb mass in the wild.

Here we set out to use quantitative trait locus (QTL) mapping in wild–domestic intercrosses to study the genetic basis of comb mass. The QTL, quantitative trait genes and causal variants for comb mass that may be detected in wild by domestic crosses may or may not segregate at appreciable frequencies in Red Junglefowl populations. The experimental intercross contains no information about ancestral allele frequencies, more than the fact that the domestic and wild parental individuals must differ for a QTL to be detected. Population genetics in natural populations would be necessary to determine the frequencies of these variants in Junglefowl populations. However, experimental intercrosses make it possible study the molecular basis of comb size variation and any potential pleiotropy, and facilitate such population genetic studies.

## Chicken bone metabolism and egg production

The abovementioned coupling of bone traits and egg production is likely not accidental. Female avian bone is special in that, during laying, it develops a kind of woven bone called medullary bone (Dacke, Arkle et al. 1993; Kerschnitzki, Zander et al. 2014). This bone type contributes little to the structure of the bone, but serves as a calcium reservoir for egg laying. It develops in response to estrogen, and indeed mammals are able to generate something similar when manipulated with high doses of estrogen (Samuels, Perry et al. 1999). All bone is in a constant state of breakdown by osteoclasts and rebuilding by osteoblasts and osteocytes. Medullary bone cycles particularly rapidly with the laying cycle. This constant remodelling in combination with the calcium demands of constant egg production may be part of the reason for the sensitivity of laying hens to osteoporosis. The genetics of bone metabolism in the chicken is therefore of practical interest, as well as part of the complex of traits involved in sexual ornamentation.

Moreover, female fecundity is a life history trait that makes up a component of fitness both in nature and in domestication. Here, as above with relative comb mass, the divergence of domestic chickens from wild chickens enables the mapping of a trait that is difficult to study within a population due to its expected polygenic architecture. Again, one will not know whether the variants and biological processes found in wild by domestic crosses are relevant to wild conditions without population genetics investigations.

## Fear and anxiety in chicken domestication

Fearfulness and related behaviours occupy a special role in domestication genetics, largely due to the research of Belyaev (Belyaev 1979; Trut, Oskina et al. 2009). Belyaev's farm fox selection experiment demonstrated that a kind of domestication could effectively be achieved solely through selection for a reduced fearfulness towards humans. Domestic chickens also differ from Red Junglefowl in their fear responses (Schütz, Kerje et al. 2004; Campler, Jöngren et al. 2009). One test situation that can be used to measure aspects of fearfulness in a reasonably objective and repeatable fashion is the open field test (Faure 1981; Suarez and Gallup 1983; Webster and Hurnik 1989). It consists in placing an animal in an open area and measuring, in this case by video tracking, how fast it moves and which parts of the arena it occupies. A more fearful chicken tends to stay around the edges of the arena (thigmotaxis) and move relatively less. The open field is sometimes regarded as an anxiety test, particularly when performed in rodent models (Archer 1973; Prut and Belzung 2003).

Fear responses involve many parts of the nervous and endocrine system. Causal variants could plausibly modify the fear response through both central and peripheral systems, and organizing as well as activating mechanisms. The hypothalamus is a reasonable choice of a measuring point given its involvement in signalling between the central nervous and endocrine systems. Also, several domesticated animals display altered hypothalamo–pituitary–adrenal activity, including chickens (Ericsson, Fallahsharoudi et al. 2014) and experimentally domesticated rats and foxes (Harri, Mononen et al. 2003; Albert, Shchepina et al. 2008). However, choosing a single point of measurement for gene expression must amount to reductive assumption about possible mechanisms.

## QTL and eQTL mapping

Quantitative trait locus (QTL) mapping is a collection of statistical methods to localize genetic variants that affect quantitative traits. The idea is to measure a phenotype in individuals and use genetic markers spread across the genome as predictors. Markers that are linked with causal variants will show association to the trait, and thus highlight regions of the genome that contain causal variants. It was developed by Soller, Brody et al. (1976) and Lander and Botstein (1989) in the context of experimental crosses. There

are also variance component methods that can handle more complex pedigrees. More recently, the availability of very dense maps of genetic markers have allowed for genome-wide association studies (Risch and Merikangas 1996) that use linkage disequilibrium in unrelated individuals instead of linkage in pedigrees. These mapping methods all have their strengths and weaknesses. A linkage design based on experimental crosses is appropriate for domestication genetics, since the objective is to compare wild to domestic chickens and find QTL that cause differences between them.

Part of the work presented in this dissertation (Papers I-V) is based on the eighth generation of an advanced intercross between wild and domestic chickens. This cross was started from one Red Junglefowl rooster and three White Leghorn hens in the 1990s (Schutz, Kerje et al. 2002). The advanced intercross design (Darvasi and Soller 1995) is a strategy to improve the resolution of linkage-based QTL mapping by accumulating recombinations over generations of interbreeding. Here, resolution means the ability to separate linked variants. An advanced intercross has higher resolution than an  $F_2$  intercross because it has seen more meioses, so that variants that initially were inherited together in blocks have had a greater chance to be separated by recombination. This work builds on the QTL mapping of comb, bone, fecundity, and behaviour traits in the advanced intercross by supplementing it with expression QTL mapping.

Expression QTL (eQTL) mapping, or genetical genomics, is the quantitative trait locus mapping of gene expression traits (Jansen and Nap 2001). The concept is simple: QTL mapping is applicable to any quantitative trait affected by genetic variants, gene expression is a quantitative trait, and hence QTL mapping can be used to locate genetic variants that affect gene expression. eQTL mapping may be undertaken with any method of gene expression measurement, but microarrays and massively parallel sequencing has allowed for whole-transcriptome eQTL maps of many organisms (see for example Brem, Yvert et al. 2002; Schadt, Monks et al. 2003; Petretto, Mangion et al. 2006; Lonsdale, Thomas et al. 2013). eQTL mapping results can be interesting in their own right. It turns out that genetic variation for gene expression is often abundant. Local, putatively cis-acting, eQTL that map back to the genomic location of the gene itself tend to be the easiest to detect, because of their relatively larger effects. Distal, trans-acting eQTL that map to different locations in the genome, are probably more abundant, but tend to have smaller effects (Noyes, Agaba et al. 2010). Because of the correlated nature of gene expression, trans-eQTL can be expected to form genomic hotspots. If a causal variant affects a central regulator such as a transcription factor, the expression of many genes should map to the location of that variant. This is indeed sometimes the case, but in practice, hotspot localization is not so easy, possibly due to the small effect size of trans-eQTL (Breitling, Li et al. 2008).

However, in addition to insights about the genetic architecture of gene expression itself, eQTL mapping can be used to search for quantitative trait genes underlying QTL. Broadly speaking, there are two ways for a genetic variant to change the function of a

gene. It may change the sequence of the protein or functional RNA to change the way the gene product works. Such variants may be discovered by sequence analysis of genes. It may also affect the expression of the gene, to change the amount of functioning gene product produced. A first proxy for studying gene-regulatory variants is eQTL mapping of transcript abundance from protein-coding genes. Concentrating on transcription of protein-coding genes does leave out several layers of gene regulation; how much is actually left out is a matter of research. Different studies have come to different conclusion about how closely transcript levels track protein levels. Some studies have investigated both eQTL and protein-level QTL simultaneously. For example, Ghazalpour, Bennett et al. (2011) found very different architectures for protein and transcript level traits, and much fewer protein-QTL in mice. In human lymphoblastoid cell lines, Wu, Candille et al. (2013) found evidence for eQTL at about half of their protein-QTL. Conversely, when using fluorescent genetic reporters on a selected set of highly expressed genes, Albert, Treusch et al. (2014) found 60% concordance between eQTL and protein-QTL. There is little doubt that there are independent genetic effects on post-transcriptional layers of gene regulation. Whether a particular variant affects gene structure, transcriptional or post-transcriptional gene regulation is an empirical question.

The gene expression measurements in this dissertation were performed either with quantitative real time polymerase chain reaction (qPCR; paper I) or with custom designed NimbleGen 12x135k oligonucleotide microarrays. In both cases, messenger RNA is reverse transcribed into complementary DNA (cDNA) with reverse transcriptase and oligo-(dT)<sub>18</sub> primers, which target the poly-A tail of mature transcripts. In qPCR, the cDNA is then amplified by polymerase chain reaction, the progress of the reaction is monitored with a fluorophore, and the amplification curve is used to measure relative expression (Livak and Schmittgen 2001). In the case of microarrays, cDNA is fluorescently labeled and allowed to hybridize to glass slides containing spots of oligonucleotide probes that match known gene sequences. The slide is read with a laser scanner, and the fluorescence intensity of the probes corresponding to a gene is taken as a measure of gene expression. In this case preprocessing (Irizarry, Hobbs et al. 2003) includes background correction, quantile normalization and summarization of multiple probes to a single probeset value. Again, the result is a measure of relative expression.

## Feralisation and selective sweep mapping

Kauai is one of the Hawaiian Islands, and like many islands it harbours interesting case studies of evolution. The free-range chickens of Kauai are abundant and fearless enough to be both an annoyance and a tourist attraction. There are two stories, or hypotheses, about the origin of these chickens. One is that they stem from feral domestics, released from chicken coops razed by storms in the 1980-90s. The other potential source is the ancient chickens brought by the original Polynesian settlers, or Red Junglefowl reintroduced in 1939. As it turns out, both of these sources, wild and domestic, appear

to have contributed to the Kauai population (paper VI). Hence, Kauai chicken population is both a feral and admixed population.

Improvements to DNA sequencing has offered geneticists and evolutionary biologists a way to get as many genetic marker as they could want, which leads not only to the abovementioned genome wide association studies, but also to whole-genome population genetics. From the perspective of domestication and feralisation studies, the most interesting application is selective sweep mapping. Empirical selective sweep mapping approaches all build on the idea of calculating some population genetic statistic in windows, or at individual loci, across the genome, and identifying the extremes. Since natural selection removes variation from the population, and linked sites hitch-hike with the selected variant, signatures of selection should manifest themselves as regions of reduced diversity (Maynard Smith and Haigh 1974). Genome scans have been performed in the chicken to search for domestication sweeps (Wong, Liu et al. 2004; Rubin, Zody et al. 2010), in domestic populations (Elferink, Megens et al. 2012; Li, Liu et al. 2012; Zhang, Wang et al. 2012; Fan, Ng et al. 2013; Gholami, Erbe et al. 2014), and in chickens adapted to altitude (Wang, Li et al. 2015). These studies have been successful in identifying some plausible candidates. One should keep in mind, though, that empirical selective sweep mapping is subject to errors and biases. Simulation studies show that selected variants sometimes leave signatures of selection in the form of reduced diversity on a genomic scale, but sometimes they do not (Teshima, Coop et al. 2006). Also, an empirical approach that uses a top percentile of the test statistic across the genome does not control the false positive rate.

Feralisation can be construed, at least at the superficial level, as the reverse of domestication: it is the evolutionary process that occurs when a population of domestic animals is put back under wild conditions outside human care (Price 1984). However, there are several wrinkles to this picture. Firstly, the feral environment may not be at all identical to the wild environment of the progenitor species. In particular, human influence may still alter the environment, for instance by affecting the habitat, predation and availability of food. Also, humans may have brought the feral animals to a different part of the world. For the chickens of Kauai, both of the above is certainly the case. Also, domestication will have affected the genetics of the population. The extent to which a certain trait that has been selected during domestication is reversible depends on the genetic architecture of the trait. If a particular locus has been fixed for a variant that is beneficial in domestic conditions but detrimental in the wild, that locus will not reverse unless a mutation or introgression occurs. When it comes to the chickens of Kauai, they may have access to wild alleles through admixture.



## Summary of papers

### I. QTL mapping of relative comb mass and the *comb1* locus

This paper reports the quantitative trait locus mapping of relative comb mass in the White Leghorn L13 x Red Junglefowl advanced intercross, as well as in a separate  $F_2$  intercross of Red Junglefowl and Obese strain White Leghorn. This resulted in five QTL regions that are replicable between at least two of these studies and the previous  $F_2$  study of the L13 cross. The largest QTL in the advanced intercross, later dubbed *comb1*, was investigated further. The confidence interval for this QTL comes down to 400 kilobases on chromosome 3, comprising a noncoding region flanked by genes *BMP2* and *HAO1*. Local eQTL mapping of *BMP2* and *HAO1* expression was performed in adult comb base tissue with quantitative PCR, and both genes showed evidence of an additive eQTL effect.

### II. eQTL mapping of comb base tissue

This paper reports the QTL mapping of bone traits, as measured by peripheral computerized tomography, in the Obese strain  $F_2$  cross, as well as a follow-up expression QTL mapping study of comb mass in the advanced intercross. There was a considerable overlap of bone QTL and previously reported comb QTL. The local eQTL study of 39 individual male comb base samples was targeted to eight comb mass QTL from the advanced intercross. eQTL mapping and trait–gene expression correlation resulted in four candidate quantitative trait genes: *HAO1*, *RGC32*, *FNDC3A* and an unknown probeset on chromosome 5. Note that for the *comb1* locus, this analysis replicated *HAO1* but not *BMP2*. It also uses the bone eQTL dataset (paper III) to investigate the potential for comb–bone pleiotropy in the comb QTL regions.

### III. QTL and eQTL for bone

Here, we performed QTL mapping of bone traits in the advanced intercross resulted in a total of 25 loci for female and 26 loci for male bone traits, with considerable differences between the sexes. Whole-genome eQTL mapping in 125 female bone samples resulted in over 6000 eQTL. This eQTL dataset found several candidate quantitative trait genes for female bone traits. Among the high confidence candidates were *DBN1*, *HSF5*, *TST3A*, and *MRSP18A*.

#### IV. QTL and eQTL for open field

This paper applies the same kind of approach, whole-genome eQTL mapping in 129 adult hypothalamus samples followed by a search for candidate quantitative trait genes, to fearful behaviour as measured in the open field test. This resulted in over 600 eQTL, and ten candidate quantitative trait genes, including *STK17A*, *GABRB2*, *SLC31A1* and hypothetical locus *LOC770352*.

#### V. QTL and eQTL for egg

This paper combines QTL mapping of female fecundity phenotypes with a reanalysis of the bone (paper III) and hypothalamus (paper IV) eQTL datasets. The results include candidate quantitative trait genes *MRPL42* and *FGF1* from bone, and *MRPL32* from hypothalamus.

#### VI. The Kauai population is admixed

In the first of two papers, the origin of the Kauai feral chicken was investigated. DNA samples from 23 Kauai chickens were sequenced using SOLiD massively parallel sequencing. Mitochondrial DNA sequences were used to construct mitochondrial phylogenies comparing Kauai samples to chicken mitochondrial sequences from databases. The presence of mitochondrial haplogroup D, as well as plumage and crowing data from Kauai chickens suggest that the population is admixed.

#### VII. Signatures of selection in Kauai differ from those in domestication

After establishing the admixed origin of the Kauai chicken population, the nuclear genomic data were used for a selective sweep scan, searching for regions of low heterozygosity. Compared to putative selective sweeps in chicken domestication, the putative feralisation sweeps were mostly distinct. We perform chromosome painting, a method where local haplotypes are “painted” by their similarity to other haplotypes. Based on chromosome painting, some of the feralisation-specific sweeps appear to be similar to Red Junglefowl and some to domestic chicken samples, indicating that the favoured alleles may be a mixture of wild and domestic alleles. Also, for a couple of sweep regions, overlap with domestication QTL suggest phenotypes that may be affected: one sweep overlaps a QTL for brooding behaviour, and another is located near genes *STK32A* and *DPYSL3*, in a chromosome 13 comb mass QTL, and that were found to correlate with comb mass (paper II).

# Discussion

## New candidates for domestication traits

This dissertation reports eQTL studies from an advanced intercross of wild and domestic chickens, and their use to analyse potential causative genes for domestication traits. This work has given rise to five lists of new candidate genes: candidate quantitative trait genes for relative comb mass, bone traits, fecundity and open field behaviour, and genes potentially selection during feralisation. Some of these genes are intuitively attractive in the light of their gene annotation and literature. Other candidates appear less obvious. For example, *BMP2* is involved in bone formation, limb development, among other processes (see review in Chen, Zhao et al. (2004)). Therefore, it is an intuitively appealing candidate for relative comb mass, even more so since *Sonic hedgehog* signalling is involved in the morphological comb variant pea comb, and also regulates *BMP2* (Laufer, Nelson et al. 1994; Boije, Harun-Or-Rashid et al. 2012). Among the bone candidates *osteonectin* (Termine, Kleinman et al. 1981) and *collagen XI  $\alpha 1$*  (Li, Lacerda et al. 1995) encode proteins with known roles in bone and cartilage, and there are also several candidates with roles related to protein glycosylation. *GABRB2*, a GABA receptor subunit, seems a plausible candidate for open field behaviour, in that GABA receptor signalling in the hypothalamus is a contributor to fear behaviour (Shekhar and Katner 1995; Zagrodzka, Romaniuk et al. 2000); see also review by Kalueff and Nutt (2007).

Most of the candidates are less known, and the literature connecting them to any organismal phenotype is sparse. This means that building on them will require more work to characterize the genes, and it also illustrates how little is known about the molecular basis of quantitative traits. One conspicuous set of genes that are well described on the molecular level, but not previously connected to organismal traits are the mitochondrial ribosomal proteins. Three mitochondrial ribosomal protein genes (*MRPS18A*, *MRPL42* and *MRPL32*) appear among the candidates for bone and egg traits. These results may warrant studies of mitochondrial function in chicken domestication.

Overall the results of the eQTL mapping studies in this thesis agree with the general patterns of detected eQTL (de Koning and Haley 2005; Breitling, Li et al. 2008; Gilad, Rifkin et al. 2008): There seems to be abundant genetic variation for gene expression. Detected local cis-eQTL were more abundant than distal trans-eQTL (even when evaluating cis-eQTL with the higher whole-genome LOD score threshold used for trans-eQTL). There were some trans-eQTL hotspots detected in the bone data, but a relation to phenotypic QTL was not obvious. Searching for overlaps between eQTL and phenotypic QTL, followed by testing for a correlation between gene expression and trait level resulted in quite a few candidates, often several candidate genes per QTL.

Viewed as a way to find causal genes, the procedure is susceptible to false positives when there are several linked genes that have eQTL and correlate with the trait. When analysed further, QTL often fractionate into several linked variants (Remington 2015), an outcome that can occur when a locus evolves through a series of mutations. However, even if repeated linked mutations are likely to occur in domestication, several genes, not belonging to the same gene family but contributing to the same organismal trait, clustering together would be surprising. Therefore, some of these QTL with many candidate genes are likely examples of linked but not causative genes. This may be because their expression is responsive to the trait, that is, their eQTL are caused by the phenotypic QTL. It may also happen that the correlation with the trait is entirely spurious, if a gene is linked with the causal gene and has an eQTL but no causal connection with the organismal trait, if the covariance between gene expression and the phenotypic trait induced by the linked eQTL is large enough.

In paper IV, a structural equations modelling approach (Aten, Fuller et al. 2008) was used on the candidate quantitative trait genes to separate these cases. The NEO model fits a causative scenario, where genotype affects gene expression to affect the organismal trait. It contrasts this with a set of alternative models where gene expression is responsive to the trait, independently caused by genotype, or affected both by genotype and trait. However, the results were mostly inconclusive, with some but not overwhelming support for the causative model in almost all cases. Complications in interpreting this analysis includes the fact that the models were fitted on gene expression data from adult birds, but the open field measurements were taken at an earlier age. This means that any signal depends on how well adult gene expression tracks juvenile gene expression. The structural equations model also deals with a single QTL at a time, when in fact these QTL are often involved in epistatic pairs. For a fuller treatment, one could fit structural equations models including all QTL and interactions (Li, Tsaih et al. 2006).

## On the generality of the results

In this dissertation, domestication has been used as a study system to map the basis of quantitative traits. In one sense, chicken domestication serves as a naturally occurring selection experiment that has induced a large divergence from Red Junglefowl in, among other traits, fecundity, sexual ornamentation, bone metabolism and fearfulness. This fact about domestication makes it possible to study traits such as sexual ornaments and life history traits, which may be more difficult to map within a population. Therefore, the interpretation of the results outside the context of chicken domestication must be somewhat guarded. What appears as a major locus in an intercross, for instance, may explain a smaller part of the variance in a Red Junglefowl population, depending on allele frequency and interactions. Similarly, conclusions about pleiotropy and linkage at the loci detected in an intercross need some qualification. Linkage

disequilibrium within natural populations generally work at a considerably shorter range than the linkage blocks even in an advanced intercross (Megens, Crooijmans et al. 2009). Tightly linked loci may be effectively pleiotropic, that is, be inherited together almost without recombination, but the physical distance for that to occur is likely much lower in most populations than in an experimental cross. On the other hand, what can be learned in experimental populations about the molecular mechanisms will still be valid.

Similarly, the potential applications of the results for breeding and medicine depend on other genetic factors. Imagine that one were to implement these results in efforts to improve the bone strength or reduce the fearfulness of domestic chickens. The desired direction of effect would most likely be from the White Leghorn allele of the intercross. One would need to know whether the breeding populations used, which are likely quite different from the L13 Leghorn line, segregates any alleles at these loci. A mapping study of the animals in question, possibly targeted to the candidate genes, would likely be needed. Related but different concerns apply if one were to use the chicken as a model for human disorders, such as anxiety or osteoporosis. The results of mining mouse and human genetics literature (papers III and V) suggest that there are cross-species similarities in these traits, but also many loci that do not overlap. For the chicken to be useful as a model, one would need to investigate these shared pathways and determine to what extent they are conserved or different in mammals and birds.

When it comes to the role of the studied traits in domestication, some may have been directly selected in the domestication and breed improvement process, and others may be more peripheral. The propensity for fear, in particular towards humans, has likely been selected in early domestication. Fearfulness has many components, and the open field test at least partially taps into a syndrome, or an axis, of fearful behaviour. Egg production, on the other hand, has been a target of artificial selection particularly during the late breed development phase. Indeed, the White Leghorns used to found the advanced intercross stem from a Scandinavian breeding programme that involved several egg production parameters. In the case of relative comb mass and bone parameters, however, it seems reasonable to hypothesize that they have increased during domestication due to correlated selection responses, possibly due to their coupling with egg production. Whether linked loci are close enough to be effectively pleiotropic depends on the range of linkage disequilibrium at the time of selection.

Linkage mapping still has its domain of use, even if it is gradually being replaced with GWAS for within-population studies. In particular, when analyzing two populations such as wild and domestic chickens, linkage mapping by experimental intercrossing is an appropriate tool. However, the intercross approach only captures variation that differs between the limited numbers of parental individuals. To address the variation within wild and domestic populations, one could use repeated linkage mapping populations. In the context of comb and bone, there are at least two different linkage studies, the L13 and OS crosses, initiated from different Red Junglefowl and White

Leghorn parentals. While the comb loci appeared quite replicable between crosses, there was much more difference in the architectures of bone traits (paper II and III). These results hint at a difference in the size of the potential set of causative loci for a trait, which is different from the realized genetic architecture, and may be called the “mutational target size” (Houle, Morikawa et al. 1996). These properties of a trait may not lend themselves to easy quantification based on this type of data, but they should affect the probability of evolutionary convergence on the molecular level and the repeatability of evolution.

## Future prospects and challenges

An appealing account of a causal variant would consist of convincing evidence for two steps: 1) that the gene in question participates in the trait, and 2) that the variant in question affects the function of the gene. While there is little doubt of the direction of causality from variant to trait, the relationship between gene expression and phenotype could go either way. Knocking down or overexpressing the gene in transgenic animals and observing the phenotype change can establish the causal effect of gene function on the trait. Generating adult stably transgenic chickens is complicated by the fact that the earliest stages of embryonic development take place while the egg is still inside the hen and not easily manipulated. However, there are methods based on injections of viral vectors into the egg after laying (McGrew, Sherman et al. 2004; Koo, Kwon et al. 2006), or making mosaic embryos by injecting transformed primordial germ cells (Kim, Park et al. 2010). However, if a proxy for the trait that can be measured in cell culture or embryos, that would probably be a smaller enterprise, since cells in culture are more easily manipulated. Finding a credible cellular or embryonic trait that reflects the organismal phenotype may be difficult. In that respect, bone traits may be the most promising. Assays for markers of bone cell differentiation, such as alkaline phosphatase in osteoblasts and tartrate resistant acidic phosphatase in osteoclasts, could be combined with RNA interference in cell culture to test the effects of knocking down candidate quantitative trait genes. Combining RNA interference or targeted gene disruption with primary cultures of osteoblast-like (Nijweide, Van Iperen-Van Gent et al. 1982) and osteoclast-like (Van't Hof, Raap et al. 1995) cells still requires some quite involved cell biology and method development.

In terms of finding the causal variant or variants, *comb1* is likely the most promising QTL of this dissertation. The 400 kb noncoding region between *BMP2* and *HAO1*, containing a potential selective sweep (Rubin, Zody et al. 2010), may be small enough to contain a manageable number of variants. Still, the problem at hand amounts to characterizing enhancers of *BMP2* and *HAO1*. One could combine the compendium of genetic variants in this region with assays of DNA–protein interaction such as DNase I sensitivity (McArthur, Gerum et al. 2001; Degner, Pai et al. 2012) or electrophoretic mobility shift (Hellman and Fried 2007). To get manipulative evidence of causality, one would then need to introduce both alleles of the potential enhancers in a reporter assay

and measure the effect on expression of the reporter gene. This assay requires a cell culture model that expresses appropriate tissue-specific transcription factors.

Sample size is a recurring challenge for all genomic studies. Power is one measure of the strength of a study design, expressed as the probability of correctly rejecting a null hypothesis when the discrepancy from the null is of a given size. Low power leads not only to a low probability of finding statistically significant results, but also leads to overestimation when significant results are found. In genetic mapping experiments of any kind, this means that genetic architectures tend to appear simpler than they actually are, because small-effect loci are missed, and those that are detected have inflated effect size estimates. Beavis (Beavis 1998) studied this phenomenon in the context of linkage mapping, and came up with the rule of thumb that around 1000 individuals is a good number for getting reasonable effect size estimates. This means that the eQTL studies here (40–129 individuals) risk overestimating the effects. This is one reason to exercise healthy scepticism when looking at variances explained and effect size estimates from eQTL studies.

When faced with low power, two possible strategies are: increasing the sample size or bringing in some other source of information. Both are at work in the field of genetic mapping. The Psychiatric Genetics Consortium, as an example from human behaviour genetics, is compiling larger and larger GWAS of human psychiatric disorders (Sullivan 2010). On the other hand, if one can get a better idea of what genes are likely to contribute to a trait, that knowledge could be brought to bear on genetic analyses. Functional background information is the rationale for candidate gene genetics, which unfortunately has a history of giving rise to associations that are hard to replicate with imprecise estimates of genetic effects (Ioannidis, Ntzani et al. 2001). There should be nothing wrong with the principle of picking a locus one has reasons to believe is involved in a trait, and testing the association between alleles at that locus and that trait. Rather, the problems likely stem from uncertainties about the size of genetic effects and what constitutes good evidence that a locus can be expected to contribute to a trait (see review by Tabor, Risch et al. 2002). Studying the basis of quantitative traits in many different species should help connect genes to organismal phenotypes, and help make informed choices of candidate genes.

### Integrating QTL mapping with population genomics

The Kauai chicken population and the advanced intercross promise the integration of genetic mapping and population genomics. In one sense, both QTL and sweep mapping studies suffer from a similar limitation: they highlight, in different ways, genomic regions that may matter to a trait or an adaptation to an environment. However, they provide little guidance about which gene and genetic variant within the region is the cause of the signal. QTL mapping may provide what selective sweeps need, namely a connection to phenotypes. However, as the chromosome 13 relative comb mass QTL

overlapping a potential feralisation sweep exemplifies, ambiguity remains. Knowing that the comb is a part of a suite of correlated traits, it is by no means clear whether this overlap is due to effects on comb mass, fecundity or bone parameters, all of which may be under selection in feral conditions.

It seems that the genomic signature of feralisation in the Kauai chicken population is very different from the signature of domestication. In clear terms: selective sweep mapping finds mostly non-overlapping regions in feralisation and domestication. If feralisation were simply the reversal of domestication, one would have expected the same regions but different alleles to be selected. This is not what the results from Kauai suggest. Rather, one or both of the following may be going on: The environment and selection pressures on Kauai may be sufficiently different from the ancestral wild environment. Alternatively, the mutational target of the traits involved may be large enough that feralisation can restore wild phenotypes through entirely different loci. In fact, for several quantitative traits in other species, the latter seems to be the case (Teotónio and Rose 2000; Qi, Liu et al. 2015).



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Dedicated to the memory of Arne Johnsson (1943 – 2011).

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