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Heritable epigenetic responses to environmental challenges

Effects on behaviour, gene expression and DNA-methylation in the chicken

Daniel Nätt



IFM Biology Division of Zoology AVIAN Behavioural Genomics and Physiology Group Linköping University, SE-58183 Linköping, Sweden

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Front cover: Red Junglefowl brooding her eggs Photo: Daniel Nätt

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To my family

Abstract

Phenotypic variation within populations is a crucial factor in evolution and is mainly thought to be driven by heritable changes in the base sequence of DNA. Among our domesticated species we find some of the most variable species on earth today. This variety of breeds has appeared during a relatively short evolutionary time, and so far genetic studies have been unable to explain but a small portion of this variation, which indicates more novel mechanisms of inheritance and phenotypic plasticity. The aim of this study was therefore to investigate some of these alternative routes in the chicken, especially focusing on transgenerational effects of environmental challenges on behaviour and gene expression in relation to domestication. In two experiments a chronically unpredictable environment induced phenotypic changes in the parents that were mirrored in the unexposed offspring raised without parental contact. This transmission was especially clear in domesticated birds. A third experiment showed that repeated stress events very early in life could change the developmental program making the birds more resistant to stress later in life. Here, the phenotypic changes were also mirrored in the unexposed offspring and associated with inheritance of gene expression. Epigenetic factors, such as DNA-methylation, could play an important role in the mechanism of these transgenerational effects. A fourth experiment showed that wild types and domesticated chickens differed substantially in their patterns of DNAmethylation, where the domesticated breed had increased amount of promoter DNAmethylation. In line with the previous experiments, this breed also showed increased transmission of methylation marks to their offspring. Conclusively, parental exposure of environmental challenges that introduce changes in behaviour, physiology and gene expression can under both chronic and temporal conditions be heritably programmed in the parent and transmitted to the unexposed offspring. Since heritable epigenetic variation between wild type and domesticated chickens is stable and numerous, it is possible that selection for favourable epigenomes could add another level to the evolutionary processes and therefore might explain some of the rapid changes in the history of the domesticated chicken.

Populärvetenskaplig sammanfattning

Tamhönan är idag världens vanligaste fågel tack vare vår utbredda kött och ägg konsumtion. Som andra domesticerade husdjur har den förändrats markant sedan den skildes från sitt ursprung, den röda djungelhönan, för ungefär 8000 år sedan. Den mest slående förändringen, som den också delar med andra husdjur, är en ökad variation bland annat på kroppsstorlek, färgdräkt och inte minst beteende. Idag anses faktiskt många av våra husdjur vara några av de absolut mest variationsrika arterna i världen; en variation som uppkommit på mycket kort tid ur ett evolutionärt perspektiv. Denna hastiga förändring kan betyda att det finns andra typer av nedärvningsmekanismer som inte är beroende av variation i själva DNA:t. I ett initialt försök att undersöka detta genomfördes därför fyra experiment. I de två första utsattes höns för en kronisk mild stress i form av en oförutsägbar hemmiljö vilket tvingade dem att förändra sina levnadsvanor och helt enkelt anpassa sig. Båda studierna visade att de beteende och genregulatoriska förändringar som skett på grund av miljön reflekterades i avkomman som aldrig själva utsatts för stressen. Intressant nog var denna miljöinducerade nedärvning tydligast hos tamhönan i jämförelse med den röda djungelhönan, vilket tyder på att själva domesticeringen kan spela en viktig roll. Det tredje experimentet undersökte en liknande generationsöverföring, men i stället för en kronisk mild stress utsattes fåglarna för korta återkommande påfrestningar under en begränsad tid väldigt tidigt i livet. Inte nog med att detta ledde till ändrad genreglering och beteendeförändring, stressade djur visade större tolerans mot stress senare i livet, vilket i sin tur överfördes till den naiva avkomman. I det fjärde och sista experimentet togs det första steget att undersöka DNA-metyleringars betydelse för nedärvning hos tam- och djungelhönan. Denna typ av s.k. epigenetisk markör är viktig för kontrollen av genernas användning i cellen och har i andra organismer visat sig ha stor betydelse för miljöinducerad nedärvning. Inte nog med att de båda hönsraserna tydligt skiljde sig i det metylerade DNA:t, tamhönan visade sig också ha större förmåga att överföra metyleringarna till sin avkomma, mycket i linje med vad som sågs i de tidigare studierna. Sammanfattningsvis har de fyra experimenten tydligt visat att det finns andra nedärvningsmekanismer hos våra domesticerade höns än vad man tidigare trott, vilket kan ha stor betydelse för såväl djurs välfärd, avel och produktion.

List of papers

The scientific substance of this thesis is based on the following four articles, which will be referred to in the text by Roman numerals:

- Paper I Lindqvist, C., Janczak, AM., Nätt, D., Baranowska, I., Lindqvist, N., Wichman, A., Lundeberg, J., Lindberg, J., Torjesen, PA. and Jensen, P. 2007. Transmission of stress-induced learning impairment and associated brain gene expression from parents to offspring in chickens. PLoS one: e364.
- Paper II Nätt, D., Lindqvist, N., Stranneheim, H., Lundeberg, J., Torjesen, PA. and Jensen P. 2009. Inheritance of acquired behaviour adaptations and brain gene expression in chickens. PLoS one 4: e6405.
- Paper III Goerlich VC., Nätt, D., Elfwing M., Macdonald, B. and Per Jensen. Transgenerational effects of early experience on acute stress reactions in behaviour, steroid hormones and gene expression in the precocial chicken. Submitted.
- Paper IV Nätt, D., Rubin, CJ., Wright, D., Johnsson, M., Beltéky, J., Andersson L. and Jensen P. Heritable genome-wide variation of gene expression and promoter methylation between wild and domesticated chickens. Submitted manuscript.

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1. Introduction

The chicken (*Gallus gallus*) is the most abundant bird species on earth today due to a worldwide annual production of 50 billion slaughtered broilers and more than a trillion eggs from about five billion commercial egg layers (Nations 2009). Due to its' efficiency in producing high quality food to low environmental costs it is predicted to play an increasing role in feeding the human world population (de Beer *et al.* 2011). The economic success of the chicken is mainly dedicated to the systematic breeding programs at international breeding companies. Precocial chicken is an ideal organism to study inheritance in, since the confounding effect of parental care can be completely eliminated. Nevertheless, it is a lie to claim that we know all aspects of inheritance in this species, because the success has not come completely without costs. Animal welfare concerns due to selection side effects, such as leg disorders and cardiovascular break down, have been raised especially against the broiler industry (reviewed by Hafez & Hauck 2005). Therefore it is not only of scientific value to use chickens as a model in inheritance studies, but also for understanding how to make it better for the billions of chickens and humans that inhabit this world.

1.1 The heritability dilemma

Without any doubt the last two centuries have turned our knowledge about the origin of species and the source of biological variation upside down. From Juan Baptiste Lamarck's ground breaking thoughts about organismal change through environmental adaptations and Charles Darwin's mechanistic explanation of this through the survival of the fittest, to the discovery of the strongest heritable elements in the living world, nucleic acids (DNA and RNA), and their significance in every biological process on this earth. To say the least, the modern day evolutionary theory is nothing but a success story for science in general and the modern synthesis of genetic evolution in particular.

Nevertheless, all heritable phenomena cannot be assigned a nucleic acid based information transfer between generations, which for decades has been a cornerstone in the modern synthesis of evolution. It has become increasingly clear that the sequence of DNA does not hold all the answers to why for example some of us show increased risks to develop certain diseases (Maher 2008) or why very closely related species can show dramatic differences in their phenotypes (Rebollo *et al.* 2010; Pai *et al.* 2011). We have become aware that it is not only the nucleic acids themselves that can preserve heritable information across generations, but also the elements that control the usage of the

information stored in the nucleic acid code. A harmful gene can never show its effect if it is turned off, and in that case it is not the gene itself that holds the relevant information about the disease, but the controller that possesses the power to switch it on or off. Similarly, all cells within a healthy human being contain essentially the same sequence of DNA. Nevertheless, the different cell types show immense functional and morphological variability, from the morphologically complex neuron to the much more simplistic egg cell. The only difference is that they use their genetic information in different ways.

The accumulation of proofs for non-genetic inheritance systems has urged many biologists to call for an 'extended evolutionary synthesis' that will 'modernize the modern synthesis' of evolution (Danchin *et al.* 2011). A new definition has been put forward that withdraws the monopoly that genes had on being the source of biological variation, where evolution now is "*the process by which the frequencies of variants*", not genes, "*in a population change over time*" (Bentley *et al.* 2004). From this standpoint this thesis begins, with an aim to explore the modernized modern synthesis in the chicken and its significance for the process of domestication, especially focusing on non-classical inheritance systems.

1.2 Domestication

Darwin was convinced that many of the answers to questions surrounding heredity and evolution lie in studying domesticated animals and plants. For instance, on the paradox of how new species can appear without good evidence of intermediate variants in our own time, he wrote: "[...] *it seems to me probable that a careful study of domesticated animals and cultivated plants would offer the best chance of making out this obscure problem.*"(Darwin 1859)

Since captive breeding has dual mechanistic properties, one part being environmental by socially taming the animal through the presence of human contact and one part being genetic through a gradual transgenerational change to better fit the captive environment, some have argued that a broad definition of domestication must be applied. Price (Price 1984) for example defined domestication as the "[...] process by which a population of animals becomes adapted to man and to the captive environment by some combination of genetic changes occurring over generations and environmentally induced developmental events reoccurring during each generation". Others make a clear distinction between the environmentally induced processes and domestication. Driscoll *et al.* (2009) sates for example that "Taming is conditioned behavioural modification of an individual; domestication is permanent genetic modification of a bred lineage that leads to, among other things, a heritable predisposition towards human association".

The earliest evidence of animals under human care comes from archaeological and genetic findings pointing out the dog/wolf as being the first domesticated animal (Driscoll *et al.* 2009). It is disputed when exactly the dog became domesticated. In the archaeological records from about 12.000 years ago there is evidence that the social bonds between humans and dogs could be so strong that they occasionally were buried in the same grave (Galibert *et al.*). The earliest finding of a morphologically dog-like wolf is dated to 32.000 years ago which suggests that the dog diverged morphologically from the wolf long before humans started to form strong social bonds with them (Germonpré *et al.* 2009). This is further supported by genetic evidence that suggests a division as far back as 135.000 years ago (Vilá *et al.* 1997; Savolainen *et al.* 2002). One popular explanation to the separation in time of the social, morphological and genetic changes is that at least in our older domesticated species domestication progressed through several phases, which suggests that subpopulations had already been 'humanised' before taken into captivity (Vigne 2011).

Even though the time points for the different phases of dog domestication are debated most agree that it was a rapid process on an evolutionary scale. It is especially rapid if we consider that most of the variation in dog breeds has been generated during the past two hundred years, during the era of intense breeding, and that the dog today is considered the most variable mammal on earth (Ostrander & Wayne 2005). Sensationally, the dog might be the most variable species, but it is not unique among domesticated animals. Sheep, pigs, goats, cattle, mice, rats, turkeys etc. all have during their domestication become much more variable (Trut *et al.* 2009). In Darwin's own words: "When we look at the individuals of the same variety or sub-variety of our older cultivated plants and animals, one of the first point which strikes us, is, that they generally differ much more from each other, than do the individuals of any one species or variety of nature." (Darwin 1859)

Also the domesticated chicken comes in a variety of breeds, spreading from the longlegged Modern Game breed to the 'fur-plumed' five toed and darkly fleshed Silkie, and from the monstrous fast growing broiler to the tiny Malaysian Serama. Archaeological and genetic findings suggest that the domestication began about 8000 years ago. It is thought to mostly originate from multiple subspecies of the Red Junglefowl of South and South East Asia (Liu *et al.* 2006), but genetic evidence has shown that at least one related species, the Grey Junglefowl, has contributed to the domestic gene pool (Eriksson *et al.* 2008). This means that the domesticated chicken is the first domesticated animal that has been proven to originate from multiple species.

1.3 Some aspects about the phenotype

The word 'phenotype' was coined by Wilhelm Johannsen (1911) exactly a hundred years ago as a resolution to the problem of distinguishing the heritable (the genotype) and the environmentally induced parts of an organism. As many have realized since then, distinguishing the effects of genes from that of environment is not easy. One of the main reasons is that organisms never stays constant, hence the phenotype is always changing due to a fluctuating environment. Nevertheless, there is always an upper and lower limit to how much organisms can change. This range in potential change is often referred to as phenotypic plasticity (Agrawal 2001; Price *et al.* 2003).

Even though most of the molecular mechanisms underpinning phenotypic plasticity are poorly understood, there are now numerous examples of adaptive phenotypic changes due to environmental causes (for examples see Jean-Christophe *et al.* and Sol *et al.* 2002). Among the more well-studied examples are the formation of predatory defence structures, like helmets and spines, in *Daphnia* species due to the exposure to predatory chemical cues (kairomones) and other stressors (Tollrian & Leese 2010). In birds and mammals phenotypic plasticity is often associated with concept of acclimatization, leading for example to increased red blood cell densities due to altered oxygen levels when ascending to higher altitudes (Storz *et al.* 2010).

Before I continue to investigate the literature behind phenotypic plasticity, and more relevant to this thesis transgenerational phenotypic plasticity, it is important to understand where on the phenotypic scale the phenomenon of behaviour is situated. The information built in the accumulated expression of genes, or in other words the transcriptome, is sometimes called the 'first' phenotype. Behaviour, on the other hand qualifies of being the 'last' phenotype, since it is most distant of the genes regulating it. It is fundamentally impossible to find purely behavioural genes, influencing solely behaviour, since the effect on behaviour is always reflecting a change in physiology or morphology. Nevertheless, bridging the gap between genes and behaviour is a necessity to truly answer Tinbergen's (1963) four questions of causation, function, phylogeny and ontogeny, hence making it one of the biggest challenges in modern ethology.

1.4 The domesticated phenotype

About 50 years ago a research team led by Dimitri Belyaev started a selection experiment at the Institute of Cytology and Genetics in Novosibirisk, Russia. Belyaev was convinced that behavioural variation was the causative element during early domestication, meaning that selecting for one single behavioural trait, namely tameability, would be sufficient for domestication to occur (Trut *et al.* 2009). He started to select untamed silver foxes

(*Vulpes vulpes*) for tameness, using a fear response score towards humans where less than 10% of the least fearful foxes were allowed to contribute to the next generation. The effect was dramatic. Just after a couple of generations aggressiveness and fear avoidance were eliminated from the selected population. Dog-like tail wagging towards humans appeared in the fourth generation and in the sixth some pups sought more actively human contact through whining, whimpering and liking behaviours (Trut *et al.* 2009). Today, after more than fifty years of selective breeding, the line show human specific communication skills not seen in any other animal than the dog (Hare *et al.* 2005).

But these were not the only effects. While the selection experiment proceeded, foxes started to breed outside their natural breeding season (occasionally even twice per year), coat colour abnormalities appeared, they got floppy ears, curly tails, widened scull, and shortened legs, tails, snout and upper jaws (Trut *et al.* 2004; Trut *et al.* 2009). The variability in the population increased hugely during the breeding. As known, increased phenotypic variation is common during domestication, but the most striking with the Russian study was that the traits that appeared where similar to those already seen in other domesticated species.

In fact, many of the traits like loss of coat colour pigmentation, curly tails, shortened legs (chondrodystrofi) and snout (brachycephaly) (Trut *et al.* 2004) and other traits like smaller adrenal glands, increased growth rate and earlier sexual maturation are so wide spread and common between different domesticated species that some have termed these deviation from the wild type as the domesticated phenotype (Price 1999).

It is tempting to speculate that these phenotypic similarities share some fundamental genetic properties. In addition, the rapid appearance after just a few generations of selection for tameness suggests that it is a simple relationship, involving selection of limited amount of genetic variation associated with relatively few genes (Stricklin 2001; Jensen & Andersson 2005). Genetic association studies, involving for example quantitative trait loci (QTL) analysis, are therefore expected to find strong overlapping associations of domesticated traits with relatively few loci, but this has not been the case. Only a small portion of the phenotypic variation observed between wild types and domesticates has been mapped to genomic locations which are spread out on a genome wide basis (Andersson *et al.* 1994; 1999; Désautés *et al.* 2002; Kerje *et al.* 2003; Wright *et al.* 2010). This indicates an additional mechanism to why we have a fast common phenotypic change in species undergoing domestication, very much in the same way Maher stresses the case of the missing heritability of human diseases (Maher 2008). Interestingly, even though cross generational changes of the phenotype during

domestication are thought to be based on genetic selection, it has been shown that much of the domesticated phenotype can appear through the right rearing conditions (Price 1999). Clark and Galef showed for example that gebrils (*Meriones unguiculatus*) reared in cages without shelter had smaller adrenals, increased growth and earlier sexual maturation than gebrils reared with access to shelter (Clark & Galef 1980).

1.5 Genetics of domestication

Even though Darwin recognised the importance of studying domesticated species for understanding evolution he also recognised some important differences to natural evolution. He especially developed the concept of artificial selection, which can be put in opposition to sexual selection, where humans instead of the animals themselves are in control of mate choice (Tiemann & Rehkamper 2009). The capacity of artificial selection to change animals is immense, illustrated for example by the already mentioned Russian fox study (Trut *et al.* 2009). Other examples can be drawn from the chicken industry where selection for non-broody behaviour in laying hens has resulted in some breeds that almost completely have lost the ability to incubate eggs and brood chicks, which is a fundamental behaviour for species survival in nature (Price 1999). In addition, during the past 50 years of intense artificial selection the broiler industry has increased the growth rate from 25 g to 100 g per day (Knowles *et al.* 2008) leading to an adult weight of about 8 kg (Goliomytis *et al.* 2003). In comparison, the Red Junglefowl weighs about 1 kg. Today a broiler chick reaches a weight of 2.3 kg in six weeks, which is ten days earlier than only 15 years ago (de Beer *et al.* 2011).

According to Price: "All of the selection imposed on captive populations that cannot be ascribed to artificial selection must be 'natural'" (Price 1999). In the initial steps of domestication, where animals for the first time are taken into captivity, natural selection of those that are best adapted to the captive environment (able to reproduce and rear their offspring) are likely to occur. For example, after just 12 months in captivity (eight generations) a wild-caught fruit fly population (*Drosophila melanogaster*) showed twice the reproductive fitness in the lab than did the original population (Frankham & Loebel 1992). Older studies by King and Donaldson, as well as by Kawahara, have shown similar results in wild-caught Norway rats and Japanese quails (as cited by (Price 1984).

Furthermore, some of the selection pressures in the wild are not present in captivity. One example is predation, which is most often dramatically decreased under human care, which has thought to relax the selection pressure of predator defence mechanism, hence leading to lower survival chances when reintroduced into the wild (Price 1984; Curio 1996). McPhee (2004) showed for example that the more generations a population of old

field mice (*Peromyscus polionotus subgriseus*) are breed in captivity, less likely they are to take cover when encountered with a predator. Furthermore, since this change was not manifested as proportional decrease, but rather a decrease accompanied by increased behavioural variability in relation to avoidance, it perfectly illustrates how relaxed selection of behavioral traits, important in the wild but irrelevant in captivity, can increase phenotypic variability under domestication.

In fact, relaxed selection has shown to directly increase genetic variability. For instance, mitochondrial DNA is extremely sensitive to energy-related selection pressures. Wild yaks (*Bos grunniens*) that roam the high-altitude and low temperature Tibetan plateau must keep a high metabolic rate to survive and therefore show less genetic variability in their mitochondrial DNA than their more inactive domesticated relatives (Wang *et al.* 2011). This difference is interesting due to the very short history of yak domestication, which again illustrates the fast genomic response to domestication. Relaxed selection on metabolic processes is probably one of the strongest effects of the captive environment, since it withdraws the activity involved in escaping predators and migrating to find food, so it is not surprising that similar results have been found in dogs (Björnerfeldt *et al.* 2006; Cruz *et al.* 2008).

Changes in selection mechanisms are not the only genetic process that differ between wild and captive environments. Two other interrelated phenomena are also thought to contribute to domestication, namely inbreeding and genetic drift. Even though there is evidence that domesticated gene pools have been backcrossed with wild genotypes (for examples see Savolainen *et al.* 2002 and Vilà *et al.* 2005), or even other species (Eriksson *et al.* 2008), the allelic diversity in domesticated breeds is thought to suffer from founder effects and bottle neck events connected to the initial domestication event and the more recent breed isolation (Lindblad-Toh *et al.* 2005). In commercial chicken breeds the situation is so severe that many have lost more than 50% of the allelic diversity in relation to non-commercial breeds (Muir *et al.* 2008) and little of the genetic diversity can be regained by crossing commercial populations since they already share common founders (Hillel *et al.* 2007). This should have an immense impact on the possibility for commercial breeds to change further. Surprisingly, so far this has not been observed in the breeding records (de Beer *et al.* 2011).

It has also been noticed that domesticated species, already before being domesticated, had some traits in common that made them more likely to become domesticated (Hale 1969). Some have argued that phenotypic flexibility is beneficial in early domestication since it would allow the animals to adjust to an array of different captive conditions (Price 1984).

Since domestication is a time consuming process from a research perspective, it is hard to investigate the genomic potential of species to become domesticated. On the other hand phenotypic plasticity in general has recently been investigated in a genomic context. For instance, Jean-Christophe et al. (2011) have compared two extremely plastic non-related arthropod species with other arthropods and found genomic properties that seem unique for the extremely plastic species, like high gene density due to local gene duplication and some epigenetic features. Gene duplication has long been thought to be an important mechanism of how species change, since it gives the possibility for one of the two paralogous genes to mutate and change its function, without harming the function of the other gene (Zhang 2003). Jean-Christophe et al. argued that since highly plastic species have more gene duplications than less plastic species, gene duplication could be a cornerstone in the creation of plasticity in these species. At least in plants gene duplication and chromosomal polyploidy are strongly connected to both adaptability and success of domestication (Dubcovsky & Dvorak 2007). In domesticated animals the diversity of other types of genetic elements, such as non coding repeats and transposable elements, could help in the generation of new varieties. For example, Lindblad-Toh et al. (Lindblad-Toh et al. 2005) showed that the genetic diversity between dog breeds constitutes to a large degree of short-interspersed nuclear elements (SINEs), which in some cases have been associated with phenotypic features such as coat colour (Clark et al. 2006) and canine diseases (Shearin & Ostrander 2010). As I will discuss later, transposable elements such as SINEs are intimately associated with generation of new genetic variability and are mainly controlled by epigenetic factors that, contrary to genetic factors, are more influenced by the environment.

1.6 Inheritance of acquired characters

Transgenerational effects independent of genetic alterations have become more and more realised as an evolutionary factor (Danchin *et al.* 2011) and could potentially play a role in the fast phenotypic changes during domestication. Heritable transgenerational effects can be seen as changes in phenotypically plastic traits in one generation that persist into subsequent generations. These parental effects can be illustrated in its extreme manifestation by the phase shifting of the Desert locust (*Schistocerca gregaria*). When colonies of this grasshopper are under low crowding conditions, individuals are shy, cryptic and nocturnal. When colony density increases, the offspring become less shy, more gregarious and start living diurnally. This change prolong for several generations until they migrate in enormous swarms (Pener & Yerushalmi 1998).

1.7 Maternal effects

Since the maternal endocrine environment is constantly influencing the developing embryo either through the placenta or through deposits in the egg, and since in the majority of cases it is the mother that cares for the postnatal offspring, it is natural to look for transgenerational effects in the mother-offspring interaction (Champagne 2011). In birds, many studies have suggested an active route between the mother and the offspring, so that the mother can manipulate the offspring phenotype to fit hers and the offspring needs. In some extent experimental findings support this idea. For example, increased levels of androgens in the eggs of several bird species have shown to affect a whole range of physiological, behavioural and other fitness related traits (reviewed by Groothuis *et al.* 2005 and Gil *et al.* 2008). In many cases androgen level correlates with the present life situation of the mother, for example if she lives in a socially demanding condition the androgen levels of the egg will increase and give rise to offspring that are more competitive (Schwabl 1997; Mazuc *et al.* 2003; Navara *et al.* 2006). Similar relationships have been seen in mothers with high social rank (Müller *et al.* 2002; Tanvez *et al.* 2008) and living in nutritional prosperity (Gasparini *et al.* 2007).

It is intriguing to put this in relation to fitness, saying that the mother makes different investments into the offspring depending on the environmental circumstance. Since we do not know how much control the female has in the interaction with the offspring this statement must be said with caution. A passive route to which the maternal endocrine environment is influencing the offspring is also possible. On the other hand, results are not in agreement in whether steroid hormones in the avian egg reflects the blood plasma levels of the mother, or not (Groothuis *et al.* 2005; Groothuis & Schwabl 2008) which is a necessity for the passive pathway to occur. Some findings suggest that the mother is at least in partial control of yolk hormone allocation. For instance, maternal corticosterone levels are many times higher than androgens in the blood plasma, while it is the opposite in the yolk (Groothuis *et al.* 2005). In addition, line selection off high and low egg testosterone in Japanese quail (*Coturnix japonica*) has shown that yolk levels are genetically independent of the maternal plasma levels (Okuliarova *et al.* 2011), indicating that yolk hormonal deposits are controlled by different genes.

Not only androgens are important for transgenerational effects in birds. Other egg components, such as antioxidants, antibodies and other hormones, have also shown maternal effects (Adkins-Regan *et al.* 1995; Royle *et al.* 2001; Saino *et al.* 2003; Groothuis *et al.* 2005; Bonisoli-Alquati *et al.* 2008; Tschirren *et al.* 2009). This is true across distant taxa as well. For example, in humans many epidemiological studies have shown that offspring with low birth weights show increased risk of developing metabolic

and cardiovascular diseases in adulthood, especially if they gain weight later in life (reviewed by Pike et al. 2008). Since birth weight is relatively independent of the offspring genotype and therefore mainly is influenced by the quality of the intrauterine environment, which further is determined by the nutritional status of the mother (Brooks et al. 1995), it was hypothesized that the mothers nutritional environment can program the development of offspring (Barker & Osmond 1986). The Barker's hypothesis which has led to the concept of the 'developmental origin of health and disease' (DOHaD) has during the past two centuries grown to become a leading opinion of the developmental process of disease. But together with the DOHaD concept a more controversial hypothesis was presented. The 'mismatch concept' states that as a consequence of adaptation by the fetus to the maternal environment, the offspring will show lower fitness if it is encountered with a different environment later in life (Bateson et al. 2004; Pike et al. 2008). This would explain why children with low birth weights that later in life gain weight will show higher frequencies of metabolic and cardiovascular disorders than those with normal birth weights that have a similar adult weight gain. The mismatch concept has been heavily criticized, even though evidence is accumulating in support of it. For example, numerous studies in multiple animal species are in line with the hypothesis (reviewed by Mcmillen & Robinson 2005) and in humans there is evidence of the opposite relationship, that high birth weight is a predictor of lower fitness if encountered with severe nutritional deprivation later in life (Chali et al. 1998). Interestingly similar observations has been seen in the already mentioned defence formation in Daphnia, where the maternal exposure to chemical traces of a predator promotes the growth of helmets in the offspring, which is associated with a fitness cost in a predator free environment (Tollrian & Dodson 1999).

1.8 Paternal effects

To date there are plenty examples of paternally transmitted environmental effects independent of paternal care, but dependent on for example male nutritional status (Pembrey *et al.* 2006; Jimenez-Chillaron *et al.* 2009), age (Garcia-Palomares *et al.* 2009; Bhandari *et al.* 2011), exposure to drugs (Abel 2004), toxins (Cordier 2008) and endocrine disrupters (Anway *et al.* 2005). For instance, human epidemiological studies have shown that food restriction in grandfathers during the pre-pubertal slow growth phase (at about 8-12 years of age) leads to increased risk of cardiovascular disease and diabetes in the grandsons but not in the granddaughters (Kaati *et al.* 2007). Phenotypic changes in the offspring due to father or grand-father dietary manipulations have also been seen in rodents (reviewed by Curley *et al.* 2011). In addition, reduced birth weights, cognitive abilities, as well as hyperactivity, have been associated with having an

alcoholic father, but only if the abusive father is the biological father which indicates that these effects are established before birth (Tarter *et al.* 1984; Hegedus *et al.* 1984). In rodent studies this paternal effect has been extended to include an array of behavioural impairments such as spatial learning impairments, aggressive behaviour as well as anxiety and increased stress reactivity (reviewed by Abel 2004). But the strongest evidence of environmentally induced inheritance in the patriline comes from studies on exposure to endocrine disruptors. For example, Skinners lab has shown that exposure to vinclozolin (an anti-adrogenic pesticide) during a critical period of embryonic gonadal development increases the risk of developing a wide variety of diseases for at least three subsequent generations, where inheritance exclusively is transmitted through the paternal linage (Anway *et al.* 2005; Anway *et al.* 2008a).

Generally there are thought to be two ways for a father to influence the development of his offspring without being in physical contact after fertilization. Firstly, he can influence the mother to change the amount of resources that she gives to the offspring and secondly he can directly affect the offspring by epigenetic factors in his sperm (Curley et al. 2011). The latter will be issued later in this thesis. Male-induced maternal effects have been studied in a wide variety of taxa, where two interrelated hypotheses have been developed and proven under different conditions: the 'differential allocation hypothesis' (DAH) and the 'compensatory hypothesis' (CH). DAH states that when the cost of reproduction is high females will increase their investments in offspring of high quality males compared to low quality males. Gilbert et al. (2006) showed that female Zebra Finches (Taeniopygia guttata) that mated with males that artificially had been made more attractive (by a red leg band) laid heavier eggs, and had offspring that grew faster with higher frequency of begging behaviours, than if they were mated with less attractive males (with green leg bands). Similar findings have been reported also in mammals, where for example female mice give birth to larger litters and more socially dominant offspring with decreased mortality when mated with males of their own choice (Drickamer et al. 2000).

As an alternative to the DAH, the CH states that females will compensate for a low quality mate by investing more resources into the offspring (Curley *et al.* 2011). For instance, Gowaty *et al.* (2007) showed in a variety of species that mating with a non-preferred partner will give offspring of lower viability, but since there were maternal compensations through an increase of the amount of eggs laid and/or offspring born, this led to a total increase of the number of offspring that eventually reached reproductive age.

The exact mechanism of how females can allocate different amount of resources dependent on mate qualities is largely unknown, but it has been shown that female birds manipulate yolk hormones deposits according to whom they mate with (Kingma *et al.* 2009).

1.9 The adaptive stress response

Stress has played a particularly important role in animal domestication due to the challenges brought upon the animals by the captive environment (Price 1999; Markel & Trut 2011). Hans Selye was the first scientist to define biological stress. He described the stress response as the 'general adaptation syndrome' (GAS), which means that organisms exposed to environmental challenges will recruit a physiological response in resistance to that challenge (Selye 1973).

The scope of this thesis is not to dwell into the extensive field of stress research and the multiple nature of stress (reviewed for example by McEwen 2007 and Koolhaas *et al.* 2011), but since environmental challenges, such as stress, have a potential to invoke adaptive responses that can affect the offspring phenotype, I must consider it through a transgenerational context.

The modern view of stress is involving the processes of homeostasis and allostasis (Selye 1955; Selye 1965; Sterling et al. 1988; Goldstein & McEwen 2002; McEwen 2007; Koolhaas et al. 2011). Stressors are environmental (e.g. heat, high population density or low nutrient availability) or internal (e.g. social isolation or psychological disorders) stimuli that threatens an animals' internal stability, or with other words its homeostasis. Generally the body responds to stress by activating the 'sympathetic adrenomedullary system' (SAM) and the 'hypothalamic pituitary adrenocortical axis' (HPA) which ultimately works to evoke the physiological and behavioural processes that make the animal escape the stressor. Since optimal environments are scarcely found, the *internal milieu* often needs to be adjusted to be able to cope or resist inescapable stressors. This process is called allostasis and was described by Sterling et al. (1988) as one of the most critical principle of physiology: "/.../ to maintain stability an organism must vary all the parameters of its internal milieu and match them appropriately to environmental demands." Purely speculative but interesting to note, the mismatch concept developed from the Barker's hypothesis in combination with the theory of allostasis and a passive perfusion of maternal endocrine mediators, permit the transmission of an adaptive stress response across generations that could affect the fitness of the adult offspring.

Behavioural and neurological responses to stress are complex. To illustrate the complexity I will only present some of the recent findings about learning in relation to stress and sex. Nevertheless, many of the ideas can be fitted to other stress related behavioural phenomena. Learning in relation to stress has previously been described with a U-shape model (the Yerkes-Dodson Law), where both under stimulated (bored or drowsy) animals and severally stressed animals will experience impaired learning performance, while intermediate stress will optimise it (Shors 2004). Lately this has been challenged since under some circumstances learning enhancements are evident in animals that been exposed to very high levels of stress (Bäumler 1994; Shors 2001; Conrad et al. 2004). Shors (2004) argued that the response is punctuated, meaning that if a threshold in stress level is met, the animal will response with either a sudden decrease or an increase in learning performance. The threshold, and whether the stress will increase or decrease performance, is dependent on multiple things such as previous experience, in utero environment and genetic background. For instance, in unstressed rats, females outperform males in classical eyelid conditioning (Wood & Shors 1998). This relationship becomes reversed when both sexes have been exposed to tailshocks, so that males outperform females. So the same stressor enhances learning in males while it impairs it in females. Studies have also revealed that the female stress response is dependent on the estrous cycle (Wood et al. 2001), where learning performance is highest during proestrous.

Since the mammalian brain has several memory systems, different kinds of stress are thought to affect different parts of the brain and therefore affect different aspects of learning (Poldrack & Packard 2003). These arguments can also be applied to sex differences, since the sexes invest differently in different brain areas (Nugent & McCarthy 2011). To explain the sex difference in learning Shors argues that sex dependent behavioural strategies, generated during the rat's recent evolutionary history, might be present (Shors 2004). In males, stress induced learning enhancements could be beneficial since they have to defend resources and territories under stressful conditions. For females, proestrous is a critical time to find a mate, hence it should be beneficial to invest more into learning and explorative behaviours during this time. But if proestrous occurs under stressful conditions (e.g. during high predatory pressure) it signals a bad timing to reproduce, hence a gain in learning abilities could be a waste of resource and should be inhibited. This explains a positive punctuated response on learning in males and a negative in oestrus females. Nevertheless, adaptive explanations like this must be used with caution. Without empirical proof in gains of fitness it might be a case of 'evolutionary fairy tailing', especially when findings in other types of learning than

classical conditioning have reported the opposite results (Conrad *et al.* 2004). Nevertheless it illustrates the problems and complexity in studying stress.

A very large amount of studies have reported transgenerational effects by maternal stress exposure or maternal injection of endogenous stress agents such as the adrenocorticotropic hormone (ACTH) or corticosteroids (for reviews see Kapoor *et al.* 2006 and Weinstock 2008). Even though the general consensus is that maternal stress leads to increased HPA activity in the offspring, much indicate, just as with the behavioral response to stress, a more complicated relationship (Matthews & Phillips 2010). For instance, Mueller and Bale (2007; 2008) found sex dependencies by showing that males exposed to maternal prenatal stress under their fetal development showed more feminized behavioural strategies and stress responses, which suggest that perinatal brain masculinisation could have been affected, which was naturally not the case in females.

But there is another response to stress that affects organisms in a broad range of taxa namely an increase of phenotypic and genetic variation (reviewed by Badyaev 2005). One of the first evidences was presented by Conrad Waddington in the 1950s. He observed that fruit flies that were exposed to heat stress during their larval stage developed crossveinless wings that would never appear during normal conditions (Waddington 1953). Not surprisingly, when he started to select on individuals with this plastic phenotypic ability the trait rapidly became more and more abundant in subsequent generations, hence suggesting high heritability of this response to larval heat exposure. More surprisingly, after some generations the phenotype appeared without the presence of the initial heat stress. Waddington explained this phenomenon by introducing the term 'genetic assimilation'. Selection on stress dependent traits 'canalizes' the genetic variation affecting the pathway contributing to the specific phenotype. Eventually, after some generations of breeding, the selected line would have assimilated enough genetic changes in the pathway to express the phenotype without the original stressor. The question was, and still is, where did this genetic variation come from?

Today there is a candidate mechanism that can explain the observations by Waddington. If a certain chaperone, Heat shock protein 90 (Hsp90), is knocked down or inhibited, it will result in increased phenotypic variability (Rutherford *et al.* 2007; Sawarkar & Paro 2010). Originally it was proposed that Hsp90 and other similar proteins works like capacitors, holding the phenotypic variation back when genetic variability increases (Rutherford & Lindquist 1998). Since stress inhibits Hsp90, stressing an organism will release genetic variability leading to new phenotypic traits that can be selected on. Lately though, Specchia *et al.* (2010) have shown that one consequence of inhibiting this heat

shock protein is increased movability of transposable elements. Transposons are genetic features, commonly thought to have viral origin, that can change position in the genome and makes up more than 40% of the 3.3 Gb human genome and much less, approximately 6-8%, of the smaller 1.1 Gb chicken genome (Wicker *et al.* 2005). Normally the movability of these elements is held back by mechanism involving for example cytosine methylation of the DNA, hence a very large proportion of our genome is constantly methylated (Slotkin & Martienssen 2007). When these control mechanisms are inhibited transposons, otherwise immobile, become active and can transpose into phenotypically important genes or their regulatory regions. The ultimate consequence is a genome-wide increased mutation rate and hence an increase of genetic variability. As I will describe in the next session, transposable elements have become increasingly important to understand genetic and phenotypic variability, not least in domestication (Lindblad-Toh *et al.* 2005; Clark *et al.* 2006; Shearin & Ostrander 2010). In addition, some heat shock proteins, including Hsp90, interact with nuclear receptors such as the corticosteroid receptor (Smith & Toft 2008), which makes it even more interesting in relation to stress.

1.10 Epigenetics: Heritable mechanism of phenotypic plasticity

The most basic criteria for phenotypic plasticity (such as stress responses) to occur is not only some sort of control mechanism of gene expression that are dependent on environmental input, but also some sort of memory system that keeps the genomic feedback stable and flexible at the same time. Many so called epigenetic mechanisms hold these properties.

Epigenetics was defined by Waddington (1940) more than half a decade ago as being "the interaction of genes with their environment, which bring the phenotype into being". Later, in the 1970s, Holliday and Pugh (1975) found DNA-methylation, and other covalent modifications of DNA, to be one of the mechanisms behind Waddingtonian epigenetic regulation. Today there is a debate concerning the correct definition of epigenetics (Griesemer 2002; Ptashne 2007; Bird 2007). Generally, two somewhat separate perspectives are present: one that defines epigenetics from a broad perspective and one that exclusively defines it from the molecular mechanisms that it involves. For instance, Goldberg (2007) defined the broad view as: "[T]he study of any potentially stable and, ideally, heritable change in gene expression or cellular phenotype that occurs without changes in Watson-Crick base-pairing of DNA". This is more or less a modification of Waddington's definition, but with heritable gene expression and cellular features as being the phenotypic level of importance. While the broad perspective are solely considering covalent modifications of DNA (e.g. cytosine DNA-methylation) and

histone modifications (e.g. histone tail acetylation) involved in gene regulation (Cuzin *et al.* 2008; Berger *et al.* 2009). As a consequence of the different views one might study epigenetics in the original Waddingtonian context by just investigating the non-genetic component of the phenotype, in the modern broad view by investigating gene expression inheritance and from the molecular perspective by investigating for example DNA-methylation.

Even though many efforts have been made to reveal the epigenetic code and find out exactly how these epigenetic marks control gene expression, much is still poorly understood. Due to the complexity of the topic the papers of this thesis mainly consider the broad perspective of epigenetic inheritance and mainly focus on DNA-methylation on a molecular level.

There are large amount of reviews explaining DNA-methylation dependent gene regulation (for some examples see Fuks 2005; Klose & Bird 2006; Jirtle & Skinner 2007; Joulie et al. 2010). Methylation in the fifth position of cytosine (5-methylcytosine) in CpG dinucleotides (cytosine followed by guanine) are by far the best studied example. It has mainly, but not exclusively, been associated with down regulation of gene expression, especially if it is present in important gene regulatory sites, such as promoter regions and other cis-regulatory sequences. It is thought to affect gene regulation mainly in two ways: by directly interfering with the ability of RNA-polymerases II to bind to promoter regions or by indirectly affecting gene expression through the interaction with methyl-CpGbinding proteins. In turn, these proteins have shown to directly affect RNA-polymerase II or indirectly affect it by modifying histones in the chromatin structure so that the DNA becomes more or less accessible for the polymerase. The patterns of DNA-methylation and histone modifications are tissue specific and play important roles in regulation of cellular differentiation. Even so, some of these epigenetic marks are dependent on interand intracellular signalling that fluctuates with the exogenous environmental context. Previously it was thought that the DNA-methylome had to be completely erased and reprogrammed for proper embryonic development, but more recent studies have shown that some epigenetic marks survive the reprogramming events and could give rise to epigenetic transgenerational effects (Morgan et al. 2005).

One of the most well characterised examples is the regulatory mechanism of the *viable yellow* allel, A^{vy} , of the *agouti* coat colour gene in mice (Dolinoy *et al.* 2007). The A^{vy} allel has a type of transposon called intra-cisternal A particle (IAP) inserted in an intronic region of the gene. Normally, without the IAP, two promoters control *agouti* by only expressing the gene in hair follicles and making the expression follow a hair cell specific

cycle (Duhl et al. 1994). This produces yellow and black banding in the growing hair which creates the typical brown coat colour of the wild type mouse. In A^{yy} mice, a strong promoter in the IAP takes over the control of the gene, and causes it to be constantly expressed not only in hair follicles but other places as well, which leads to a totally different phenotype with yellow coat colour, obese appearance and increased risk for tumour genesis (Dolinoy et al. 2007). So far it is quite strait forward: a transposable element with a strong promoter invades the gene and overrides the fine-tuned natural control of its expression. But as discussed before, the activity of 'jumping' genetic material like viruses or transposons are kept in control by genetic silencing mechanism, such as DNA-methylation and/or chromatin modifications (Slotkin & Martienssen 2007). So normally the IAP of A^{vy} allel should be silenced, but this is not the case. In fact, A^{vy} is an example of a so called metastable epiallele (Rakyan et al. 2002), where the methylation status of the IAP is very much varying between cells and individuals. The consequence is that an isogenic A^{vy} mouse will show different phenotypes (ranging from brown to yellow and normal to obese) depending on the methylation status of the IAP promoter. Whitelaw's lab also showed that yellow mothers, with a hypomethylated IAPpromoter, was more likely to have offspring with a hypomethylated IAP-promoter, hence this epigenetic mark somehow survives the embryonic reprogramming events and can be inherited (Morgan et al. 1999). Later studies, have been using the agouti viable yellow phenotype and a related epiallel dependent phenotype, the axin 1 fused $(Axin1^{Fu})$ that causes a kinked tail, as instruments to measure the environmental impact on the methylome for example through parental diet (Wolff et al. 1998; Cropley et al. 2006; Waterland 2006), alcohol consumption (Kaminen-Ahola et al. 2010) or other environmental manipulations (reviewed by Rosenfeld 2010), showing a direct pathway from the parental environment to the epigenetic inheritance of DNA-methylation marks.

Interestingly, it has been reported that the *star* gene, which causes a typical coat colour alteration in many domesticated species, has shown irregular segregation in offspring from crosses between heterozygotes, indicating epigenetic silencing of one of it alleles in a similar way as the *agouti viable yellow* and *axin 1 fused* (Belyaev *et al.* 1981; Trut *et al.* 2009).

That both DNA-methylation and histone-modifications show transgenerational inheritance is not surprising, since there is extensive cross-talk between them for example through the interaction of DNA and histone methyltransferases (Cedar & Bergman 2009), polycomb-group proteins (Vire *et al.* 2006) and methyl-CpG-binding proteins (Lan *et al.* 2010). The problem is to figure out if any of them is the carriers of information in the cross-generational transmissions seen in metastable epiallels or if they are just the

products of another carrier system. Interestingly, some findings have shown that the methylation of IAP of the $A^{\nu\nu}$ allele is in fact erased during the embryonic reprogramming events, which suggests that another carrier must be present to make this mark reappear later in development (Blewitt *et al.* 2006). Some of these carriers could help to explain paternal transgenerational effects independent of paternal care and male induced maternal-effects, which also have been associated with DNA-methylation and histone modifications (Curley *et al.* 2011). Promising candidates are different kinds of non-coding RNA particles (such as siRNA, piRNA and miRNA) which are abundant in sperm cells and in some case have proven to modify DNA-methylation/chromatin patterns often in relation to the silencing of transposable elements (Rassoulzadegan *et al.* 2006; Johnson *et al.* 2011).

Broadening the perspective makes us realize that there are several other types of epigenetic carrier systems in the broad sense. For instance, it has been shown that the quality of maternal care can be the carrier of epigenetic information by affecting the DNA-methylation status of nuclear receptors, such as the glucocorticoid receptor (Champagne 2011). Nuclear receptors are potent transcription factors regulating gene expression and have been shown to induce behavioural associated DNA-methylations (Weaver et al. 2007). The glucocorticoid receptors themselves are known to be involved in mediating epigenetic information through histone remodelling (John et al. 2008). This means that the influence by maternal hormones on the developing embryo and the exposure of postnatal offspring to parental behaviour, could also be seen as carriers of epigenetic information that ultimately might affect DNA-methylation and the chromatin configuration. Jablonka and Lamb (2007) have even argued that evolution should be seen through four dimensions: genetic (e.g. mutations), epigenetic (e.g. DNA-methylation), animal tradition/learning (e.g. maternal care, social learning) and symbolic learning (e.g. book reading in humans). In fact, all dimensions transmit heritable information between generations and could potentially be mediators of gene expression inheritance.

Heritable epigenetic responses to environmental challenges in the chicken

2. Aims

The overall aim of this thesis was to explore transgenerational epigenetic inheritance in the chicken and its significance for the process of domestication.

Paper I.

This paper investigated behavioural and gene regulatory changes as a result of exposure to an unpredictable environment, mainly aiming at the differences between the wild type Red Junglefowl and the domesticated White Leghorn breed and the subsequent effects on their unexposed offspring.

Paper II.

The second paper investigated the effects seen in Paper I more thoroughly by hypothesising that the phenotypic changes could have an adaptive basis, both in the exposed parents and in their unexposed offspring. In addition, the inheritance of gene expression was also more thoroughly investigated.

Paper III.

In Paper I and II the effects of a chronic unpredictable environment were investigated. Paper III changed the perspective slightly and looked instead on the effect of parental early life stress and its effect on behaviour, gene expression and corticosterone stress reactivity in the offspring.

Paper IV.

All the previous papers showed positive gene expression inheritance of acquired changes due to environmental challenges that indicate some sort of epigenetic inheritance. Results from Paper I also suggested that the domesticated genotype transfers gene regulatory information more efficiently than the wild type. In the last paper one of the first attempts to explore the epigenome (DNA-methylation) of the chicken and its relevance for inheritance was taken, with the aim to decipher some parts of the mechanisms that transfer gene regulatory information between generations, within and between breeds.

3. Methods

3.1 Animal material

Three different chicken breeds have been used in this thesis. In Paper I and IV, where the domestication effect was investigated, the Red Junglefowl (Fig. 1a) together with a domesticated White Leghorn breed were used. Even though the exact genetic statuses of these populations are not known, both have been kept in isolated populations of 20-40 individuals for more than ten generations, and hence, due to genetic drift and mating between more and more related individuals, should be considered relatively inbreed. The Red Junglefowl population was brought from Thailand to a Swedish Zoo in 1993 and taken into research facilities in 2000 (Schütz *et al.* 2001). The White Leghorn population originates from an experimental line that been selected for high egg production since the 1970s (Liljedahl *et al.* 1979) and brought into maintenance research breeding at the same time as the Red Junglefowl (Schütz *et al.* 2001). Plenty of phenotypic traits differ between these two breeds, such as plumage colour, egg and body mass, age of sexual maturation, and a vide range of social, foraging and fear related behaviours (for more information see Jensen 2006).

Changing focus to purely investigate transgenerational effects within domestication, Paper II and III exclusively used a commercial hybrid egg layer (Fig. 1b): the Hy-Line W98 (2008). This hybrid, which is a widely used bird in the industry, originates from



Figure 1. Two of the three breeds that have been used in this thesis. In (a) Red Junglefowl female with her chicks in an outdoor enclosure at the research facility at Vreta, Linköping. In (b) a commercial Hy-Line W98 female tested in the foraging arena of Paper II at the Götala research station in Skara (photos: Daniel Nätt).

great grandparent inbred lines bred at the Hy-Line International facilities in Dallas, USA. These pure lines are evaluated through a very strict breeding programme that involves selection criteria both within line and in crossbred progenies. Over 30 different traits divided into five categories are continuously monitored: production, egg quality, efficiency, animal well-being and reproduction (O'Sullivan 2011). In relation to the other two breeds the Hy-Line becomes larger, lays larger eggs and reaches sexual maturity earlier.

3.2 Measuring behaviour

Together there are twelve different tests performed within this thesis. I will not go into details on all these tests in this frame story since they are well described in the papers or elsewhere. Generally, the tests have been used as a toolbox for characterising the phenotypic changes in the parents and the significance of these changes in the offspring, always in connection to stress response and the underlying effect by domestication.

The tests can be divided into four themes within the context of measuring the effects of stress and fear. [1] General activity: open field (Paper III-IV), tonic immobility (Paper III), aerial predator (Paper IV), fear for human (Paper IV). [2] Learning: spatial (Paper I), associative (Paper III). [3] Exploration: foraging strategy (Paper II), food preference test (Paper II), novel object test (Paper III). [4] Social behaviours: competition (Paper I-II), dominance (Paper III) and social reinstatement (Paper III).

With only a few exceptions, behavioural observations have been done using video cameras, assisted by either behavioural sampling software (Noldus Observer®) or digital video tracking software (Noldus EthoVision®).

3.3 Hormone analysis

In Paper I, II and III hormonal analyses were performed. While the first two papers concentrated on evaluating the possibility of egg hormones to mediate epigenetic transgenerational information (Paper I only looked at corticosterone and Paper II at corticosterone, testosterone, dihydrotestosterone, oestradiol and androstendione), Paper III investigated both egg hormones (testosterone and oestradiol) and blood plasma corticosterone.

For all hormonal analyses immunoassays, either radioactive (e.g. RIA) or fluorescent (e.g. DELFIA), were used. The main principle of hormone immunoassays is that a hormone specific radioactive or fluorescent labelled antibody binds to the hormone and by the fluorescence intensity the concentrations can be estimated in relation to a standard curve.

The biggest problem with this technique is the specificity of the antibodies, which can unintentionally bind to other confounding substances (such as other hormones).

For instance, Rettenbacher *et al.* (2009) have shown that the antibodies used for detecting corticosterone cross-react with progesterone and its metabolites. Even though these antibodies only have affinities of 1-2% to these gestagens in relation to corticosterone, it can substantially influence the results in yolk hormone studies, since the level of progesterone metabolites are very high in the yolk while corticosterone is low. This means that studies that used immunoassays for investigating yolk corticosterone might in fact have studied gestagens. It also means that the RIA-based corticosterone measurements in the egg yolk of Paper I and II of this thesis might not be as reliable as we first thought. In Paper III, where we knew about the cross-reactivity problem, we skipped the egg yolk corticosterone analysis.

3.4 Gene expression microarrays

In all four papers some sort of genome wide gene expression microarray were used comparing the gene regulatory changes within and across generations. Microarray is a surface based detection method where some sort of microscopic printing technique is used to apply thousands, or even millions, of micrometer sized probe spots onto for example a glass or silica surface not larger than a thumb. These rows of tiny probes, all with different binding preferences, are used to detect the levels of specific molecules in a sample. In gene expression microarrays the probes are made of single stranded nucleotide chains, each specifically designed to bind (or hybridise with) a certain modified massager RNA molecule. Since the RNA are labelled with a fluorescent dye it can be detected after binding to the probe and through its fluorescence intensity levels the relative concentration can be estimated. The big advantage with microarrays is that more or less the whole transcriptome can be investigated in a very small sample with just one analysis. The big problem is that it generates enormous amount of data that can be difficult to handle and interpret.

In Paper I and II a custom made cDNA microarray, developed at the Royal Institute of Stockholm (KTH) within the framework of a chicken research cooperation (The Wallenberg Consortium North), was used to compare the birds living under the unpredictable environment with the controls, as well as their respective offspring. cDNA microarrays use clone libraries of complementary DNA as probe material. The KTH chicken array uses a dual colour detection system, where two kinds of samples (e.g. the actual sample and a common references sample) are labelled with two different dyes (green and red) and later hybridised to the same array. By doing this the actual sample

can be compared to the references sample through a relative colour scale. For example, if the probe spot colour is completely green the actual sample is in abundance and has competed out the reference sample; if it is yellow the actual and the reference sample are in the same concentrations; if it is red the reference sample is in abundance. By using the same reference sample on every array (but of course using different actual samples) the relative concentrations of the actual samples can be estimated indirectly in relation to the reference sample. The KTH cDNA chicken microarray contains approximately 14000 cDNA probes, meaning that almost the same amount of different gene transcripts can be analysed at once.

In Paper III and IV the GeneChip® Chicken Genome Array that can analyse about 33000 unique transcripts and are commercially available from Affymetrix Inc. were used. This array uses another microarray technique than the KTH cDNA microarray, where short (25 bp long) synthetically made oligonucleotides instead of cDNA are used as probes. Without using a reference sample, but instead using single colour detection, the Affymetrix arrays can measure the absolute concentration of transcripts by measuring the single colour intensity at each probe spot. The limitation of the array is that due to its short length the probes are predisposed to non-specific binding to other transcripts, as well as having problems to cover whole transcripts (which sometimes can be several kilobases long). Affymetrix have solved the problems in two ways. Firstly they have introduced sibling probes that are identical to the real probe except for just one mismatch. By this they can measure the degree of non-specific binding and withdraw those probes that show high non-specific binding from the analysis. Secondly, there are several full matched probes for each transcript, or with other words a probe set, which together gives the signal that is translated into transcript concentration.

Analysing microarray data is not easy since it suffers from two fundamental statistical problems: [1] the simultaneous testing of thousands of probes and [2] finding small effects in enormous often very noisy datasets. The problem with multiple testing is that the more tests you perform the higher is the risk that any of them will be a false positive (type I error). This is often solved by different kind of p-value corrections, basically making it harder to reach significance threshold. One of these corrections is the FDR adjusted p-value that has been used in all papers of this thesis (Hochberg & Benjamini 1990). The second problem relates to the first since when you make it harder for statistical significance to emerge, you simultaneously increases the risk for small effects to disappear by becoming false negatives (type II error). Since there is a dilution effect in transgenerational studies (the parental effect will be diluted by the signals from the offspring's own environment) effects could be expected be small. The problem is often

solved by decreasing the number of spots included in the experiment by using different kinds of filters that take away biologically irrelevant and bad quality probes. In Paper I and II a rigorous filter incorporating several filtering criteria (such as spot saturation, spot size and background comparisons) were applied, where about 50% of all spots were filtered away before the analysis. In Paper III, since the unfiltered FDR-adjusted p-values were heavily skewed, a variance filter was applied that took away the probe spots with lowest variance, making the distribution of p-values less skewed (Bourgon *et al.* 2010).

The best way to limit the type I and II errors would be to repeat the experiment in another group of samples. One of the powers of transgenerational studies is that this can be done within the experiment just by choosing relevant subsets of genes. In Paper I the most differentially expressed genes of the comparisons in the parents were correlated with the offspring results. This got rid of a lot of noise from treatment irrelevant genes in the offspring that otherwise would have compromised the analysis. But since some genes in the parents are expected to be false as well, we realised in Paper III and IV that there is an even better subset to base the correlations on, namely the overlap of the topmost differentially expressed genes in both generations. This automatically limits the contribution of both false positives and negatives in both generations. Hence in a very simple manner we limited both the multiple testing problem as well as decreased the risk of having small effects drown in the noise of the full experiment. To my knowledge, we are the first that are doing this in transgenerational studies.

3.5 DNA methylation tiling array

In Paper IV yet another kind of array technique was used to in large scale measure DNAmethylation at promoter regions, mainly for the purpose to explore differences between the Red Junglefowl and the White Leghorn. Here we constructed a custom made so called tiling array together with the Roche NimbleGen, Inc. While gene expression microarrays use probes based on gene transcript sequences, tiling arrays use genomic sequences taken from species reference databases. In our case we tiled the sequence of 3623 promoter regions, defined as 3.25 kb downstream and 7.25 kb upstream of a gene start site. Tiling implies that we put synthetically made oligonucleotide probes (50-75 bp long) on the array, with an approximate spacing of 100 bp, resulting in about 110-120 probes per promoter. The long oligonucleotide probes minimise the problem of specificity as was the case for the Affymetrix array (see above text). By isolating DNA and use a technique called Methylated DNA Immunoprecipitation (MeDIP) we efficiently enriched a portion of our sample with methylated fragments. This was done by letting an antibody with high affinity to 5-methylcytosine bind to the methylated DNA fragments. After immobilising the antibody/methylated DNA complex and washing away the unmethylated DNA fragments, we ended up with a low concentration sample of only methylated DNA-fragments. After whole genome amplification the sample was, in relation to the originally sample, enriched with DNA fragments.

Just as with the dual colour cDNA microarray, we then labelled the enriched sample and hybridised it to the custom made tiling array, together with the original sample that was not enriched with methylated DNA fragments (but still contained methylated DNA). The crucial point is that the enriched sample carrying one colour will compete for binding to the probes with the original sample labelled with a different colour. This leads the enriched sample, highly biased with methylated fragments, to outcompete the original sample and give a stronger colour signal at those probes where DNA-methylations are present. Since each probe relates to a specific region in the genomic sequence, the exact location of methylation in the 3623 gene promoters tiled on the array could be detected.

4. Summary of Papers

Paper I.

Separate groups of White Leghorns (WL) and Red Jungelfowls (RJF) were chronically exposed to unpredictable (UL) or predictable (PL) light rhythm, beginning five weeks after hatch. After fourteen weeks of exposure a subset of animals from each group underwent a spatial learning test showing that both the WL and the RJF birds living in the UL environment had spatial learning deficiencies in comparison to the PL groups. Offspring to each group were hatched and reared under the PL environment. In their fifth week the offspring were tested in a similar spatial learning test as their parents, where the WL, but not the RJF, showed similar spatial learning deficiencies as their UL parents in relation to the offspring of the PL parents. Next, a microarray experiment was performed to evaluate the treatment effect on hypothalamic brain gene expression and correlate the expression profiles across generations. This showed that the WL, but not the RJF, transferred brain gene regulatory information across generations, meaning that the differences in transcription acquired through the environmental challenge was somehow carried over to the offspring.

Paper II.

To further dissect the phenotypic changes and the transgenerational effects in Paper I, the treatment protocol was replicated in a commercial hybrid commonly used in the egg industry. Two main hypotheses were constructed to make a preliminary assessment of the adaptive value of the changes associated with UL exposure. Firstly it was hypothesised that in an environment with unpredictable food access, birds will show more conservative as well competitive feeding strategies, simply because they cannot predict when the food will be accessible again (they need to take the opportunity to ease their hunger). Secondly, the adaptive changes in the parents will be transmitted to the offspring and thereby suggestively prepare the offspring for the parental environment. To test the hypotheses two different behavioural tests were used. First a foraging arena where birds could freely forage among three potential food sources: one readily available, one highly desirable but hidden in wood shavings, and one false source, looking just like the desirable source but containing nothing but wood shavings. As predicted the UL treatment shifted the feeding strategy towards a more conservative on. This was also the case for the female offspring, but not the male, suggesting a sex specific transgenerational effect. Furthermore, while there was only a numerical difference in parental competitiveness, both sexes in the offspring of UL parents were as predicted more competitive towards a common food source than the offspring of PL parents. Also differences in growth and disease

susceptibility appeared only in the offspring. The gene expression analysis was not as clear as in Paper I, both when it came to the number of significantly differentially expressed genes and the correlation between generations. Nevertheless there were significant transgenerational correlations and immune genes emerged as strong candidates for the transgenerational effects seen. Interestingly, UL female also showed an elevation of oestradiol suggesting a possible mediator of the transgenerational effect.

Paper III.

A new treatment protocol was introduced in Paper III. Here a presumably stronger stress package (social isolation, food restriction and temperature change) than the UL was repeatedly applied to a group of chicken during three weeks very early in life. This early stress treatment (ES) was then evaluated through the behavioural phenotype, glucocorticoid stress reactivity, and brain gene expression later in life and in the next generation. Clear effects in all three categories were seen in the parents, where ES birds were for example better in solving an associative learning task and had decreased glucocorticoid reactivity compared to unexposed controls. In addition stress related genes, such as early growth response 1 (EGR1) and corticotropin releasing hormone receptor 1 (CRHR1), were significantly affected, but only when analysed in the context of acute stress through a 30 min restraining period. Interestingly EGR1 was upregulated after acute stress in all groups investigated (males, females, ES and controls), while CRHR1 was upregulated only in ES males. A sex difference was also seen in the transgenerational effect, where the male offspring to ES parents showed a similar corticosterone response and gene regulatory change as their parents. Unexpectedly, the physiological and gene regulatory changes in the offspring were not manifested in the offspring behaviours, but only seen as an increased growth rate later in life, much similar to Paper II. Also similar to Paper II a weak yolk hormonal effect was present, where oestradiol and testosterone were elevated in eggs of ES females.

Paper IV.

In Paper I-III environmentally acquired correlated transgenerational effects of gene expression and other phenotypes were evident, suggesting some sort of epigenetic inheritance mechanism, either context dependent through the influence of for example yolk hormones, or germline dependent through the direct survival of epigenetic marks over embryonic reprogramming events. Paper IV was therefore one of the first attempts to explore the epigenome of the chicken, investigating the potential for transgenerational epigenetic inheritance in this species in the context of domestication. Again WL and RJF were used, where breeding pairs of clearly different phenotypes within each breed were

allowed to hatch offspring. Much of the phenotypic variation in the parents was inherited by the offspring. With a combination of a gene expression microarray and a DNAmethylation tiling array we saw stable inheritance of breed differences on both gene expression and DNA-methylation, but also within breed stability of gene regulatory inheritance. Interestingly, the pattern of DNA-methylation was stably inherited in WL, but disrupted in the RJF, suggesting a fundamentally different transgenerational epigenetic inheritance mechanism in the wild type. This was supported with data showing that the promoters epigenetically affected during domestication had generally increased their methylation levels. Possibly this difference in inheritance capability of epigenetic marks may be the crucial difference that made the WL, but not the RJF, acquire and transmit environmental information across generation in Paper I.

5. General Discussion

5.1 Behavioural genetics

In all four papers large scale gene expression experiments were performed focusing on changes of transcription in the hypothalamus or a combination of the hypothalamus and thalamus. Even though, the nuclei of the thalamus and hypothalamus of birds and mammals look rather different and can be found on different relative locations, many of their functions are thought to be conserved between taxa (Kuenzel & van Tienhoven 1982; Richard *et al.* 2004; Kuenzel & Jurkevich 2010). These parts of the brain were chosen since they play a significant role in the stress response, where for example the neurons of the paraventricular nucleus of the hypothalamus (PVN) produces corticotropin releasing hormone (CRH) which is an activator for the HPA-axis (Kuenzel & Jurkevich 2010). Since gene expression often is tissue specific, differential gene expression in the brain could indicate possible genetic pathways regulating the behaviours investigated in the experiments. Therefore, throughout the papers of this thesis many possible behavioural gene candidates have been proposed.

For instance, in Paper I differential expression of the *brain derived neurotrophic factor* (*BDNF*) is a strong candidate of the spatial learning impairment of the UL parents and their offspring, since this gene codes for a neuropeptide which has a wide effect on neural plasticity, which is very important in for example new memory formations (reviewed by Cowansage *et al.* 2010). A recent chicken study has shown that the promoter to *BDNF* can be hypermethylated by heat stress under certain embryonic phases which seems to affect the activation of the gene by the same stress later in life (Yossifoff *et al.* 2008). Future studies should therefore investigate the role of the epigenetic regulation of *BDNF* on the transgenerational effects of stress seen in Paper I.

In Paper II, immune genes were differentially expressed. One of these genes, the *immunoglobulin lambda-like polypeptide 1 (Ig light chain, IGLL1)*, was also affected in some of the treatments of Paper I and Paper III (and even in Paper IV). Besides being a strong candidate for the decreased disease susceptibility of the offspring to UL parents in Paper II, it has been shown that other immune genes, like the rodent *MHC class I*, also play important roles in neural plasticity (Shatz 2009). So far though, the neural function of the homolog to *MHC class I* in the chicken has not been confirmed. Nevertheless, the reason why *IGLL1* is highly dynamically expressed in brain tissue, generally associated with an up-regulation in stressed birds, indicates that this gene has a neural stress related function. Since immune genes have very complicated expression mechanisms, granting

some of them the ability to generate the extreme diversity of antibodies in the vertebrate immune system, the task to decipher its exact role in behavioural processes of stress and domestication probably lies far into the future.

The best example of a candidate gene system that should be relatively easy to investigate in the near future is the candidates presented in Paper III. Here the well characterised corticotropine releasing hormone receptor 1 (CRHR1), coding for one of the main controllers of the HPA-axis, was affected in males but not in females. A similar sexually dimorphic relationship has been seen before in for example humans (Wasserman et al. 2008; Heim et al. 2009), but not in relation to the early growth response 1(EGR1) and early life stress. EGR1 is an immediate early gene that functions as a transcription factor and is suggested to play an important role in neural plasticity, together with for example BDNF (Knapska & Kaczmarek 2004). Identifying possible 'cross-talk' between ERG1 and *CRHR1* could be very important for understanding the sex-dependent physiological and behavioural responses to stress not only in birds, but maybe even in humans. Differential CRH and ACTH (Adrenocorticotropic hormone) administration could help to investigate the relationship between the receptor and the corticosterone response in Paper III, since the ACTH stress response is relatively independent of the CRHR1 receptor. Furthermore, since these two genes were found differentially expressed only in the transition between unrestrained baseline and restrained birds, hence the gene expression difference was acquired during only a 30 min acute stress, it indicates that the CRHR1 promoter of ES male birds was already sensitised before the restrain treatment. This suggests some sort of epigenetic mechanism through which the CRHR1 gene was 'pretriggered' for a positive transcriptional signal. Investigating DNA-methylation and/or histone modifications, as well as the binding potential of the ERG1 transcription factor to the CRHR1 promoter, could further reveal the mechanisms underlying the sex and treatment differences in Paper III.

5.2 Gene expression inheritance and cross generational adaptation

In all four papers of this thesis, positive significant transgenerational correlations on differential gene expression have been presented, meaning that if a relevant subset of genes is up-regulated in parents in relation to a reference group, the very same genes tends to be so also in the offspring. To date very few studies have investigated large scale gene expression inheritance in this way, where the majority of studies concentrate on small scale candidate gene approaches (e.g. Cameron *et al.* 2008) or only consider significant differences within advanced generations (e.g. Anway *et al.* 2008b). In all papers of this thesis a substantial amount non-significant transcripts were correlated between parents and offspring, which indicates a high number of false negatives.

Limiting the view to only look at significant genes could therefore be misleading in large scale gene expression inheritance studies.

Nevertheless, we have shown that genome wide gene expression correlations are a powerful way of measuring inheritance, which is particularly illustrated in Paper IV, where within breed differences artificially selected over just one generation and with few significant manifestations, are almost as strongly correlated across generations as differences between breeds with many significant manifestations that diverged thousands of generations ago.

In Paper I-III, the only difference between the groups of interest (treatments) and the reference groups (controls) is an exposure to a challenging environment that has forced the treated groups to change, or if so preferred, adapt to the present environmental situation. It is evident from our results that not only do chickens acquire changes in gene regulation by sensing the environment, but it is not unusual that they also project this change onto the next generation. The evidence becomes even stronger when considering phenotypic changes on other levels, such as behaviour, where whenever there are significant differences in the offspring, they are just reflections of similar differences in the parents; the inverted relationship is very seldom observed. Therefore, there is no doubt that at least in the chicken there are biological mechanisms that under many situations seem to prepare the offspring for the parental environment. The question is just are they true mechanisms by themselves, increasing the possible fitness of the offspring by foreseeing the most probable juvenile environment, or are they just parts of something greater, an unavoidable by-product of something so evolutionary important that it is worth risking the fitness of the offspring. Especially in avian yolk hormone studies, an over interpretation of the female control mechanism might be at hand, where words such as 'maternal differential allocation' or 'maternal offspring investment', are too often used without even knowing if the mother can adjust yolk hormone levels independently of her own physiology (Groothuis & Schwabl 2008). Nevertheless, androgen, oestrogen and progesterone producing cells are present in the cell layers of the follicular wall that is the interface between the female circulation and the growing oocytes (Groothuis & Schwabl 2008). This anatomy would make independent control possible. The genetic independence between hormonal levels of maternal blood and eggs, as been reported in quail (Okuliarova et al. 2011), strongly supports this idea, but further studies looking at fitness outcomes under different maternal/offspring environmental schemes are needed to evaluate the adaptive significance of any maternal control mechanism.

One initial step in investigating the adaptive properties of a transgenerational effect was taken in Paper II. This paper illustrates how good hypotheses based on logical thinking about the possible outcomes of robust behavioural and physiological systems could help to investigate adaptive components in phenotypic traits, without provoking too much 'evolutionary fairy telling'. Feeding strategies under hungry unpredictable conditions are such a system. In Paper II, hungry birds living under unpredictable conditions risk a potential cost if not finding food before the opportunity disappears, in relation to birds living under predictable conditions. This is why they are expected to show high competitiveness and a preference to forage on readily available food when having a chance. Many studies have investigated the effect of unpredictable food availability, but to my knowledge none has done so without eliminating the possibility of confounding factors that may be beneficial to the group that are exposed to unpredictability. Unpredictable food availability in nature almost always comes with a choice: stay with your strategy and wait for better times, or migrate and explore new possible food sources. Not surprisingly, some studies have shown that unpredictable food availability promotes more explorative behaviours, boldness to novelty and associated learning enhancements (e.g. Pravosudov & Clayton 2001; Bridge et al. 2009; Chapman et al. 2010). Farm and laboratory housing often eliminates the possibility to explore new places and food resource, hence leaving the animal with only one choice: stay and adapt. In Paper II birds restricted to their home pens and exposed to unpredictable food availability responded with a less explorative and competitive foraging strategy, which were reflected in the behaviour of the offspring even though they never experienced the parental treatment. So were the offspring 'pre-adapted' to their parent's environment? To fully answer this question offspring fitness components must be estimated under the same environmental conditions as their parents were living in. This must also be applied to answer the same question in the transgenerational reflections of Paper III.

5.3 The epigenetic dissection of domestication

Paper IV is one of the first attempts to explore the epigenome of the chicken, and the first to my knowledge that puts it into relation of inheritance. A contemporary study by a Li *et al.* (2011) mapped the DNA-methylation through genome-wide MeDIP-sequencing and found similar mechanistic patterns as in many other species, such as CpG island and promoter hypomethylation, as well transposable element and repetitive sequence hypermethylation and down-regulation of gene expression. On the other hand, they found no evidence of parent-of-origin genomic imprinting which are present in both mammals and plants (Reik & Walter 2001). They also investigated differences between the Red

Junglefowl and a domesticated broiler breed, but since they only used one bird from each breed, no conclusions independent of individual variation can be drawn. In Paper IV, on the other hand, we used four adult Red Junglefowl and four adult White Leghorn birds, and tracked the differences in pools of their respective juvenile offspring (in total twelve chicks from each breeding pair). This is a much more powerful design to find true breed difference, as well as stable differences that are independent of age and generational circumstances. We showed that over 140 promoters were significantly differentially methylated between breeds, with a majority being hypermethylated in the White Leghorns, indicating that domestication directly has affected the epigenome by *de novo* methylations in the domesticates. The effect of this can only be speculated at this point, but since DNA-methylation is a key player in transposable element control and that the loss of this control recently has shown to release genetic variability (Specchia et al. 2010) it is possible that it affects phenotypic plasticity and maybe even evolvability (Johnson & Tricker 2010). Even though we are far from rejecting or confirming the hypothesis that the fast phenotypic change seen under domestication is due to transgenerational epigenetic inheritance, we have shown that, at least in the White Leghorn breed, genes affected by domestication are selectively epigenetically altered. Interestingly, we did not find an association between down-regulation of gene expression and hypermethylation of the same genes, as was reported in Li et al. (2011). This is not an uncommon observation though (for examples see Carone et al. 2010 and Lister et al. 2009) and could indicate a more complicated relationship between DNA-methylation and gene expression, for example through interactions with methyl-CpG-binding proteins (Lan et al. 2010). The dissimilarities between our results and Li et al. could be an experiment dependent difference. They associated absolute levels of gene expression with absolute levels of methylation, answering the question: if a gene is highly expressed will it show low amount of methylation? So far our focus has been on correlating expression differences between breeds with differences in methylation, hence answering the question: if a gene is upregulated in the White Leghorn in relation to the Red Junglefowl, will it show an inverted relationship on DNA-methylation? Our result indicates that this is not the case, hence suggesting a different role for DNA-methylation in the domestication of the chicken. In our data, the unconditioned exploration of the chicken methylome, as Li et al. did, is still pending analysis and will most probably be published in a separate paper in the near future.

Surprisingly though, at some of the methylated sites presented in Paper IV very few CpG dinucleotides were present. This suggests either that we have retrieved the signal from other types of cytosine methylations (Lister *et al.* 2009) or that the antibody commonly used in MeDIP protocols cross-reacts to cytosine independent DNA-modifications. In

these cases the causal relationship with gene expression are principally not known yet. Interestingly, a recent finding has shown a larger proportion of hemi-methylated DNA (methylation on only one of the DNA strands) in chickens compared to other animals (Shao-Qing *et al.* 2007). Since DNA-methyltransferase I (Dnmt1) uses hemimethylated CpG dinucleotides as templates for maintaining methylation on both DNA strands, it could indicate that other types of DNA-methylations independent of Dnmt1 proliferation are abundant in the chicken genome.

5.4 Perspectives: The long road to Lamarckian inheritance

Behavioural epigenetics is a rapidly growing research field that already is playing or is predicted to play a role in the vast amount of behavioural systems studied to date (Champagne & Rissman 2011). This is also true for behavioural studies related to animal welfare, since early life challenges in the animal industry, such as social stress, maternal isolation and prolonged physical restraint, could have profound effects on the stress reactivity later in life and even on subsequent generations. The potential for epigenetics to have an impact on animal welfare is dependent on the possible future findings of relatively stable epigenetic marks controlling welfare related genes (such as CRHR1) and how well these findings can be incorporated into animal managements. Unfortunately, developmental aspects of management practices have already been realised for example in good human animal interaction and the value of an enriched environment (Fraser 2009), but mostly been neglected in large scale industrial systems. Nevertheless, especially in the chicken industry where relatively few great grandparental birds contribute to a very large amount of the world's chicken population, considering transgenerational epigenetic effects could have a huge impact on animal welfare, as well as disease susceptibility and many production traits.

While evidence is accumulating that epigenetics plays an important role in inheritance, the long term evolutionary significance is still rather obscure. From my perspective there are at least four possible routes for epigenetics to work in an evolutionary context: [1] a control mechanism for transgenerational phenotypic plasticity; [2] a releaser or generator of genetic/epigenetic variation due to environmental effects; [3] genetic assimilation of transgenerational phenotypic plasticity; [4] true Lamarckian inheritance that adaptively produce environmentally acquired and completely new varieties that can be transmitted across generations.

[1] As described in the introduction there are plenty of examples of transgenerational phenotypic plasticity, best illustrated in the transgenerational phase shifting of the Desert locust (Tollrian & Dodson 1999). Unfortunately most prenatal stress studies in mammals

only concentrate on the detrimental effects on the offspring, not asking if the offspring phenotype reflects the environmentally acquired phenotype of the parent. This is similarly true for the majority of egg hormone studies, but here the focus on the evolutionary significance of a possible maternal investment in the offspring is diminishing the interest in heritability. In Paper I, II and III we have clearly shown inheritance of phenotypic plasticity, from which some already have evoked the 'Lamarckian ghost' to explain the results. Even though Lamarckian inheritance is at the horizon inheritance of phenotypic plasticity can very well be explained with other mechanism. Again the phase shift of the desert locust illustrates this perfectly. Due to environmental cues this grasshopper goes from being nocturnal, shy and cryptic to become diurnal, social and gregarious, but even though this change is sustained for several generations, it is nothing but a pre-programmed shift in the regulation of genes. In this process only latent phenotypic variation is released based on already established genetic variation. The same thing might apply for reflective transgenerational effects of stress, since stress responses could be seen as gradual shifts in the phenotype caused by a pre-programmed change in gene expression.

[2] But stress might in fact be different. As also mentioned, environmental challenges could epigenetically promote release or *de novo* generation of genetic variation through for example inhibition of heat shock proteins (Sawarkar & Paro 2010). Whether or not this is the case in the studies of this thesis is still to be addressed, but heat shock proteins and other immediate early genes, such as the *EGR1*, are present on the top gene lists of all four papers. Investigating phenotypic variance like McPhee did on old field mice (McPhee 2004), but more in relation to stress responses in domestic and wild type breeds could reveal completely novel information about domestication.

[3] Even though *de novo* genetic and epigenetic variation is probably not responsible for phenotypic shifts in the exposed generation, it might contribute to new variation in the subsequent generations, and by the process of canalization it could lead to genetic assimilation. Interestingly, this combination of transgenerational phenotypic plasticity [1] and heat shock protein related increase of variation [2] would partly mimic true Lamarckian inheritance but without invoking other than Darwinian mechanisms. Stress induced phenotypic plasticity in the parent that increases genetic variation in the offspring and simultaneously leads it into a developmental pathway that would promote those genetic changes that are necessary for the shifted offspring to change even further in same direction, is a perfect explanation to Waddington's observation when he selected on crossweinless wings in *Drosophila* more than half a century ago (Waddington 1953). But as realised this speculative mechanism needs lot more research to prove its case.

[4] For true Lamarckian inheritance to occur *de novo* adaptive variation in the parental phenotypic response to the environmental challenges must happen. Since *de novo* mutations are commonly believed not to have other than detrimental effects in the adult organism (such as cancer), the source of such variation is probably epigenetic. The question if new phenotypic variation within an individual can arise through *de novo* generation of epialleles which later would be inherited by the offspring is far from answered and will probably be the golden egg of modern 'Lamarckian' biologist for many years to come, potentially influencing our understanding of such diverse things as pathology, ecology, ethology and animal welfare. Or as Mr. Charles Darwin himself said:

"[...] I think there can be little doubt that use in our domestic animals strengthens and enlarges certain parts, and disuse diminishes them; and that such modifications are inherited." (Darwin 1859)

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6. Conclusions

This thesis concludes:

- 1. Transgenerational effects of parental environmental challenges are common but complex in the chicken, being dependent on multiple factors such as type of challenge, sex and genetic background.
- 2. Since it often happens in a reflective manner, where gene regulatory, endocrine, morphological and behavioural phenotypes of the parents match the phenotypes of the offspring, it is a type of inheritance.
- 3. And since selection during domestication has changed the ability to inherit some of these environmentally acquired traits and more specifically changed the methylation levels of genes involved in the domestication process, epigenetic inheritance might be an important factor contributing to the transgenerational effects seen.

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