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REVIEW

Adding 'epi-' to behaviour genetics: implications for animal domestication

Per Jensen*

ABSTRACT

In this review, it is argued that greatly improved understanding of domestication may be gained from extending the field of behaviour genetics to also include epigenetics. Domestication offers an interesting framework of rapid evolutionary changes caused by well-defined selection pressures. Behaviour is an important phenotype in this context, as it represents the primary means of response to environmental challenges. An overview is provided of the evidence for genetic involvement in behavioural control and the presently used methods for finding so-called behaviour genes. This shows that evolutionary changes in behaviour are to a large extent correlated to changes in patterns of gene expression, which brings epigenetics into the focus. This area is concerned with the mechanisms controlling the timing and extent of gene expression, and a lot of focus has been placed on methylation of cytosine in promoter regions, usually associated with genetic downregulation. The review considers the available evidence that environmental input, for example stress, can modify methylation and other epigenetic marks and subsequently affect behaviour. Furthermore, several studies are reviewed, demonstrating that acquired epigenetic modifications can be inherited and cause trans-generational behaviour changes. In conclusion, epigenetics may signify a new paradigm in this respect, as it shows that genomic modifications can be caused by environmental signals, and random mutations in DNA sequence are therefore not the only sources of heritable genetic variation.

KEY WORDS: Methylation, Epigenetics, Gene expression, Transgenerational effects

Introduction: behaviour, genes and evolution

Animals respond to and affect their environment, including other animals, by means of their behaviour. The muscular system is one instrument by which they can manipulate their surroundings, and the responses are mediated by a set of emotional and motivational mechanisms. The evolutionary and ecological function of behaviour, and the way in which it is controlled and develops, is the central issue of the science of ethology. All these aspects are the under influence of genes (Bendesky and Bargmann, 2011).

In fact, the genetic basis for behaviour variation may be one of the most important discoveries of ethology (Jensen, 2006). The insight, growing since Darwin, that animals do not come into the world as blank slates, but are genetically pre-programmed for complex actions, forms the foundation for understanding how and why behaviour has evolved under natural selection. In consequence of this fact, alleles causing behaviour that promotes life-time reproduction will be favoured during evolution.

Linköping University, IFM Biology, AVIAN Behaviour Genomics and Physiology Group, 58183 Linköping, Sweden.

*Author for correspondence (perje@ifm.liu.se)

This review will consider the evidence for direct genetic influences on behaviour, and its importance in domestication, a special case of evolution. It will then be expanded to include novel aspects from epigenetics. Animal domestication constitutes a well-defined evolutionary process, occurring during a brief time span, allowing in-depth considerations about the mechanisms involved. It will hopefully become clear that the field of animal welfare, which, among others, concerns the way in which animals respond to environmental challenges associated with stress, will also benefit substantially by including new perspectives from epigenetic insights. In this review, the term 'stress' will be used loosely to describe a wide range of responses to environmental challenges. The concept is often restricted to situations where there is a clear activation of the hypothalamic-pituitary axis, coupled with a behavioural loss of control (Koolhaas et al., 2011), but in this review it will also be used to describe situations in which such responses can be assumed, but are not necessarily measured.

At the heart of evolutionary development and domestication resides the idea that selection acts on the available genetic variation. But what causes genetic variation, and is it really the only evolutionary valid substrate for selection? According to neo-Darwinism, as crystallized in the 20th century, this would indeed be the case (Futuyma, 1997). This view is based on the assumption that genetic information flows only in one direction, from DNA via RNA to proteins and phenotypes, and that genetic variation can therefore only occur as a result of (random) DNA mutations. In other words, experiences and environmental signals should not be able to modify the way in which the genome works. This has obviously been proven wrong by research over the last 20 or so years (Guerrero-Bosagna, 2012; Jablonka et al., 1998).

The 'new' view that has entered biology is epigenetics. The concept was originally coined by Waddington (Holliday, 2006) and its development has been excellently reviewed (e.g. Jablonka and Raz, 2009). Today, it is normally used to describe a variety of chemical modifications to DNA that affect gene expression and are mitotically, and sometimes meiotically, stable (Holliday, 2006). Some of the epigenetic modifications are sensitive to environmental input. Hence, epigenetic insights offer a novel way of viewing the interaction between the environment and the genome, and hence evolution. Although the research is still in its infancy, it is likely that the findings will prove to represent a shift in paradigm in biology.

Domestication as an evolutionary case story

Animal domestication provides a handy model for evolution, where behavioural responses to selection can be assessed directly, as, in most cases, the ancestral species are still available for comparison (Price, 2003). In addition, often the specific selection pressures affecting the domestic populations are relatively well known. For example, in dogs, selection has originally been for breed-specific behaviour variation, and during the last few hundred years also for

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appearance, while for most farm animals, selection has focused on reproductive traits and growth (Larson et al., 2012; Mignon-Grasteau et al., 2005). For most wild populations, neither the ancestors nor the specific selection pressures are known, which complicates deeper analysis.

Domestication can be defined as the process whereby populations of animals change genetically and phenotypically in response to the selection pressure associated with a life under human supervision (Jensen and Wright, 2013). One can picture domestication as an evolutionary process whereby populations adapt to a life with humans. For example, weaker stress responses towards humans and higher tolerance towards variations in social grouping can both be beneficial responses in this situation.

During domestication, specific natural selection pressures, such as predation and starvation, are relaxed and human selection for preferred traits is intensified. Furthermore, traits that are correlated to the ones selected may change as a consequence of genetic linkage or pleiotropy; for example, increased relative gut length in fast-growing broilers (Jackson and Diamond, 1996).

To understand how behaviour is affected by domestication, we compared free-ranging domesticated White Leghorn laying hens with their ancestors, the Red Junglefowl (Schütz, 2001), and found, for example, that leghorns are less active and exploratory, and display less social behaviour. This might indicate an adaptive response to life under human protection, where food is abundant, predation is less of a risk and crowding is common. Other experiments show that some traits also appear to have evolved as specific adaptations to human interactions. For example, dogs are better than wolves at responding to human cues such as pointing and gazing, and they can learn and understand large numbers of spoken words and put these into categories (Miklósi, 2008).

It has been suggested that many of the behavioural, morphological and physiological changes observed in domesticated animals may originally have developed as correlated side effects of tameness, perhaps because genes with various pleiotropic effects regulate fearfulness. This is to some extent supported by the famous selection experiment on farm foxes (Trut et al., 2009). Although selected for increased tameness only, many foxes developed, for example, loss of pigmentation, faster ontogenetic development, curly tails, floppy ears and compressed jaws, all commonly associated with domestic dogs. In a similar experiment on rats, Albert et al. (Albert et al., 2009) also found correlated effects, but in this case the evidence rather indicated that they were caused by the combined effects of several physically linked genes. Focusing on Red Junglefowl (ancestors of all domestic chickens), we found significant genetic correlations between fear of humans and, for example, foraging and exploration, and hatch mass (Agnvall et al., 2012). All these studies indicate that selection for tameness, which normally would imply reduced stress sensitivity, is likely to cause correlated behavioural responses, and that the genetic architecture of a few linked or pleiotropic genes may affect a large part of the domesticated phenotype. Exploring the nature of such genetic effects may greatly increase our understanding of evolution.

Principles of behaviour regulation and genetics

Before going into depth with the genetic and epigenetic mechanisms involved in behavioural effects, we need to consider the machinery of behaviour regulation and its components. Behaviour occurs on very different levels of organisational complexity, and a simple definition states that behaviour is ‘all observable processes by which an animal responds to perceived changes in the internal state of its body or in the external world’ (Barnard, 2004).

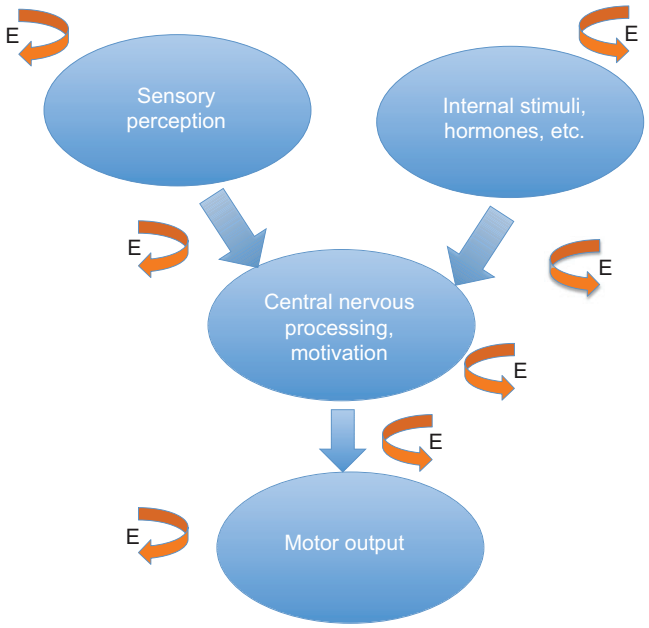

Fig. 1. A simplified scheme of how behaviour, defined as motor output, is controlled by different physiological processes. The scheme also indicates how every part of the behaviour control system can be modified by epigenetic changes (arrows marked with ‘E’).

The behaviour control system relies on input from sensory systems, monitoring both external (e.g. olfactory and visual) and internal stimuli (e.g. hormones), which is integrated in the central nervous system (CNS). Also, the CNS itself generates activity without sensory input, causing, for example, diurnal or annual behaviour rhythms. The actual behaviour is usually a product of muscular activity, and therefore depends on the organisation of joints and muscles in the organism (Fig. 1).

Genes can therefore affect behaviour by means of two main classes of mechanisms (Jensen, 2014). The first covers irreversible effects occurring mainly during embryo development, which direct how the nervous system is wired, organised and assembled. The design of sensory organs, muscular apparatus and the skeleton and joints also falls in this category and produces non-reversible limits for behaviour plasticity. The other way genes may affect behaviour is by reversible effects, involving dynamic changes in gene expression. Genes involved in the control of hormones, receptors or neurotransmitters can modify behaviour throughout life. For example, modifications in song repertoires of song birds, as well as yearly variation in singing activity, are governed by cascades of changes in gene expression in specific parts of the brain (Clayton, 1997). Similarly, annual breeding cycles and fluctuations in social patterns depend on variations in the expression of relevant genes (Robinson et al., 2008).

Proof that genes affect behaviour in a probabilistic or deterministic manner comes from classical genetic crosses, as well as selection experiments, and direct genetic manipulations, such as knockouts, with associated behaviour measurements. For example, *Drosophila* selected for short or long mating times for seven generations had a mean mating speed of 3 versus 80 min (Manning, 1961). Scott and Fuller found several simple Mendelian patterns of inheritance of differences in behaviour between dog breeds (Scott and Fuller, 1965), and knockout mice lacking the gene coding for the neuropeptide oxytocin show a reduced ability to remember the identities of other individuals (Ferguson et al., 2000).

Many studies also show significant heritability for behavioural traits. For example, in Swedish flatcoated retrievers, heritability (h) for different behaviours varied between 0.1 and 0.5 (Lindberg et al., 2004). Based on results in a hunting–retrieving test, h^2 was 0.49 for 'excitement', and 0.28 for 'willingness to retrieve'. Similarly, German shepherd dogs showed high heritability for 'activity' (0.53) and 'contact' (0.42) (Ruefenacht et al., 2002). In an experiment examining the genetic basis of domestication, we selected birds with either high or low fear of humans for three generations from an outbred population of Red Junglefowl (ancestors of modern chickens) (Agnvall et al., 2012). We found moderate to high heritability of some traits closely related to domestication: 0.17 for 'fear of humans' and 0.32 for 'distance moved in open field' (a measure of general fear). Findings such as these emphasise that complex behaviour traits have a significant genetic component and are liable to selection.

Behavioural genes

Measuring the quantitative contribution of genetic components is of course only a first step on the route to understanding the complex interplay between genes and behaviour. The ultimate purpose of any study is normally to locate causative genes and their mutations, and to use this to outline the complete pathway in which a particular genetic variant can be associated with a certain behavioural phenotype. As of yet, this has rarely been achieved. However, the development of the genetic toolbox is fast, and a number of so-called 'behaviour genes' have been identified. This term is used to denote genes that have a clear and strong effect on behaviour variation, although the mechanisms by which this is exerted are rarely known in detail.

Top-down methods for finding genotype–phenotype associations take their starting points in some known phenotypic variation and then use various mapping methods to associate this variation with specific genomic regions, and ultimately with specific genes and mutations. Bottom-up approaches instead start with some known genetic variation and use different methods to pinpoint the phenotypes associated with it.

Top-down methods have been widely used in behaviour genetics, most typically in association studies, such as quantitative trait locus (QTL) analysis, or genome-wide association studies (GWAS). However, bottom-up methods have become more powerful with the decreasing cost and increasing efficiency of DNA sequencing methods. This allows researchers to scan entire genomes for genetic variation between populations and thereby find candidate regions with a low degree of heterozygosity, indicating selection for one particular sequence variant. When a certain population has a fixed haplotype at a certain locus, or at least a very low degree of heterozygosity, this is referred to as a selective sweep. Mapping of selective sweeps can, for example, reveal important loci that contain genes selected during domestication, although it rarely offers more than hypotheses as to the phenotype targeted by the selection.

It should be emphasised that the mapping methods outlined can just as well be applied to search for connections between epigenetic variation and specific phenotypes. I will return to this aspect later in this review.

Although somewhat limited by usually not being accompanied by knockout or knock-in experiments, top-down methods have led to the discovery of some of the most well known behaviour genes, for example the gene *npr-1* in *C. elegans*, which causes either foraging in groups (social foraging) or solitary foraging (de Bono and Bargmann, 1998). Furthermore, *Drosophila* larvae show a distinct dimorphism in their foraging pattern, referred to as 'sitters' and 'rovers', and this is controlled by the genotype at the *for* locus (Osborne et al., 1997), a cGMP-dependent protein kinase gene. In chickens, we mapped the propensity to be victims of feather pecking, a detrimental behavioural disorder common in the industry, to a genetic polymorphism in *PMEL17* (Keeling et al., 2004). This gene encodes a melanocyte-specific protein, essential for maturation of melanosomes, and the identified domesticated mutation inhibits all expression of black pigment in the bird. It turned out that the mutation not only causes a white plumage, and reduces the risk of being the target of feather pecking, but also has pleiotropic effects on exploration and aggression (Karlsson et al., 2010; Karlsson et al., 2011).

Yet another example of a successful top-down analysis is the identification of mutations in the promoters of the arginine vasopressin receptor (*AVPR1a*), which has important effects on pair-bonding and social behaviour in a range of species, including humans (Donaldson and Young, 2008; Walum et al., 2008). In chickens, the gene is located on chromosome 1, and we have found that it is probably involved in some of the domestication-induced modifications of social behaviour in this species (Wirén et al., 2013; Wirén and Jensen, 2011).

Bottom-up methods have been explored more recently, but an interesting example of its potential is provided by the resequencing of several populations of domesticated chickens (Rubin et al., 2010). This identified a large number of selective sweeps, most likely the result of selection during domestication. The strongest signal was found over the thyroid-stimulating hormone receptor gene (*TSHR*), where domestic chickens carry a 9 bp insert mutation in the transmembrane region and it is quite likely that this sweep stems from mutations selected because of their putative effect on reproduction. Research on this is currently going on in my lab.

Epigenetics – gene expression and regulatory mechanisms

Until very recently, behaviour genetics had mainly been concerned with identifying DNA polymorphisms associated with behaviour variation. The general assumption was that only such genetic factors matter in an evolutionary context, as they can be transmitted through the germline to the next generation, and thereby cause transgenerational changes. However, the overwhelming bulk of recent research clearly demonstrates that most evolutionary changes, not least with respect to behaviour, appear to be associated with regulatory mutations (Andersson and Georges, 2004; Hofmann, 2003). These do not affect protein structure, but rather when and to what extent a particular protein is expressed.

It is therefore no surprise that interest has been growing in understanding gene regulation in relation to phenotypic variation. Some of the regulatory variations are clearly explained by pure genetic mechanisms, but many are due to chemical modifications, which do not depend on DNA sequence variation. As outlined in the Introduction to this review, this is the subject of epigenetics (Richards, 2006).

Large phenotypic differences can be the result of differences in gene expression profiles, for example sex differences within a species. Of course, the genomes of males and females of the same species are extremely similar, except for one sex chromosome (e.g. Y, which is only found in male mammals, and W, which is found in female birds). Nevertheless, the genome-wide gene expression profile differs greatly between the sexes, and this is at least partly the result of epigenetic sex differences (Ellegren and Parsch, 2007). In mice, sexually dimorphic gene expression is already present before embryonic hormone secretion starts (Dewing et al., 2003) and in chickens, hundreds of differentially expressed genes have been

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found in the brains from female and male birds of both ancestral Red Junglefowl and domesticated White Leghorns (Lee et al., 2009; Nätt et al., 2014). Many of the chicken genes were associated with stress responses (e.g. the corticotrophin releasing hormone binding protein, *CRHBP*, and the GABA receptor subunit *GABRA2*), which is particularly suggestive, as the sexes differ markedly in their emotional and stress-related responses, as measured in standard tests such as 'open field' and 'fear of human' (Nätt et al., 2014).

Also, between species and populations, gene expression profiles vary extensively. For example, in a comparison of the brains of humans and chimpanzees, hundreds of genes were found to be differentially expressed (Zeng et al., 2012), and many of them were related to neural development. In an ambitious cross-species analysis, Albert et al. (Albert et al., 2012) searched for brain cortex-related gene expression signals common to domesticated mammals. Although the study did not reveal such a common signal, it was clear that the differences in gene expression appeared considerably larger than the sequence differences between pairs of domesticates and their wild ancestors. This suggests that gene expression variation may be more important in speciation than variation in coding sequences. Similarly, we compared the hypothalamic gene expression differences between ancestral Red Junglefowl and domesticated egg laying chickens and found hundreds of differentially expressed genes (Nätt et al., 2012). These were over-represented in selective sweep regions, indicating that during domestication, there may have been extensive selection of gene expression profiles considered favourable by humans.

Epigenetic mechanisms: what causes differences in gene expression

Gene expression is obviously an important key to understanding evolution and speciation, and most research so far has focused on two mechanisms affecting this: methylation of cytosines in CpG positions and chemical modification of histones, such as methylation and acetylation (Richards, 2006).

CpG-dinucleotides are not uniformly distributed in the genome, but tend to accumulate in certain regions, referred to as CpG-islands, which are ubiquitous in gene promoters. Methylation of cytosines in promoters normally causes downregulation, or even silencing, of a certain gene (Richards, 2006). Also, histone modification can affect transcription, and to increase the complexity, such modifications are often closely related to cytosine methylation (Richards, 2006). Targeting of sites liable to epigenetic modification is to some extent controlled by small interfering RNAs, which therefore are also important epigenetic factors.

The overall individual specific pattern of epigenetic marks – the epigenome – can be conceived of as the orchestration of the genome. It controls the timing and intensity of gene expression and therefore is crucial to understanding phenotypic differences between populations. As behavioural variation often correlates with variation in gene expression (Hofmann, 2003; Jöngren et al., 2010), epigenetic mechanisms should be important in behaviour regulation as well. However, the research area is still novel, and relatively unexplored. A recent review paper quotes only a total of 96 papers dealing with behavioural epigenetics (Lester et al., 2011).

Richards, in his seminal review, divided epigenetic marks into three different classes, termed obligatory, facilitated and pure (Richards, 2006). The obligatory ones are strictly determined by genotype, so a particular locus in a specific cell type will always carry a typical epigenetic signature, and, in humans, over 80% of the genome-wide variation in methylation patterns depends on differences in genotype (Gertz et al., 2011). In chickens, we found that some genes that were differentially methylated when comparing brains of domesticated chickens and ancestral Red Junglefowl maintained their methylation differences over eight generations of inter-crossing (Nätt et al., 2012). Some of the methylation differences showed a Mendelian inheritance pattern, which strongly indicates that they were under genetic control.

The so-called pure marks are those that are independent of genotypes outside the affected locus, while facilitated epigenetic marks depend in a probabilistic manner on genotype. From the perspective of transgenerational effects, facilitated and pure epigenetic variants are of highest interest, being most likely to show dynamic responses to external events.

In order for epigenetic mechanisms to play any role in transgenerational effects on behaviour and consequently in behavioural evolution, they need to possess a number of traits. Firstly, they must be able to respond in an adaptive fashion to environmental stimuli, causing gene expression profiles to change. Secondly, gene expression variation must be related to variation in behaviour, allowing animals to alter their behaviour in response to such stimuli. Thirdly, such environmentally induced modifications should be able to survive gametogenesis and re-establish a similar gene expression profile in the relevant tissue of individuals in later generations. Hence, they need to be heritable.

I have elaborated in detail on these aspects in previous reviews (Jensen, 2013; Jensen, 2014), and will expand on some of the aspects in the rest of this paper as well, but, mostly, it appears that all these processes do in fact occur: epigenomes respond in a non-random, often adaptive way to environmental signals, they cause behavioural changes and they are often inherited. This means that the interaction between the genome and the environment is far more dynamic and complex than previously thought and our view on domestication and evolution may have to be rather drastically revised.

Epigenetic responses to environmental signals

A number of relatively recent reviews have summarised the growing bulk of research showing that epigenetic marks are highly responsive to various environmental cues (Curley et al., 2011; Kappeler and Meaney, 2010). Stimuli related to stress, aggression or reproduction can cause transient changes in brain gene expression mediating proper physiological and behavioural responses. Sometimes this can even cause life-long modifications in behavioural response patterns. The ways in which epigenetic changes can affect behaviour are ubiquitous (Fig. 1). Genes involved in all aspects of the behavioural control system, from sensory input to motor output, can be regulated by, for example, promoter methylation, causing a modified response pattern.

Meaney and co-workers studied the long-term consequences of different types of early maternal care in rats (Meaney, 2001). Pups raised by mothers performing more licking and arched-back nursing had an increased resilience to later stressful events. This was mediated by modified expression of genes involved in the stress response system, as well as in genes governing hippocampal synaptic development. The quality of maternal behaviour affected hippocampal DNA methylation, which was associated with alterations in histone acetylation and binding of the transcription factor *NGFI-A* (nerve growth factor-inducible protein A, also known as *egr-1*, *krox-24*, *zenk* and *zif-268*) to the glucocorticoid receptor (GR) (Weaver et al., 2004).

The effect was limited to experiences during the first weeks of life, which coincides with the time when maternal behaviour shows most individual variation. Furthermore, more than 900 genes were

differentially expressed according to the level and quality of the early maternal care experienced (Weaver et al., 2006). Early maternal care in rats has also been shown to affect the expression levels of the oestrogen receptor *ERα* in the medial preoptic area of the brain, related to increased methylation of the *ERα1b* promoter. The epigenetic differences persisted into adulthood, and could be reversed by cross-fostering (as well as by pharmacological inhibition of histone acetylation), which provides compelling evidence that it is the actual experiences of different maternal quality that cause the effects. In humans, the GR *NR3C1* showed a different methylation pattern in hippocampus samples from suicide victims who had a history of childhood abuse (McGowan et al., 2009). The abused persons had a very similar downregulation of NGFI-A binding to that of the less careful rat mothers.

Epigenetic effects may occur before birth. Prenatal alcohol exposure has been shown to cause hypermethylation and downregulation of the epigenetically regulated allele *Agouti Viable Yellow* (Kaminen-Ahola et al., 2010). Altogether, the evidence is compelling that experience during different life periods, including before birth, can cause lasting modifications in epigenetic marks.

Stress and fear – central components of domestication

Identifying the central selection pressures is crucial for understanding possible epigenetic involvement in evolution. Early in domestication, it is obvious that animals with a low propensity of showing fear towards humans and being stressed by human proximity must have been favoured. Hence, selection for reduced stress responses may have been the primary evolutionary pressure for any domesticated species. As already noted, it has also been suggested that many of the features typical for the domesticated phenotype could be passive, correlated responses to such a reduction in stress and fear (Trut et al., 2009).

Stress occurs when the control systems of an animal are over-ridden (Koolhaas et al., 2011), and it is usually associated with increased levels of circulating corticosteroids, mainly cortisol (or corticosterone) (Toates, 1995). Steroids easily pass cell membranes, and bind to nuclear receptors (for cortisol, the GRs) in the cytoplasm, and the complex is transferred to the nucleus, where it binds to specific DNA sequences, acts as a transcription factor and modulates gene expression downstream (Sapolsky et al., 2000). GR-binding sites are widely distributed in the genome, so corticosteroid activation during stress causes a cascade of effects on gene expression (Hunter, 2012). As described earlier in this review, epigenetic modifications of the GR can persist for a long time and alter the stress responses and behaviour of an animal throughout life, caused by inhibition of NGFI-A binding to its consensus sequence by DNA methylation (Weaver et al., 2007).

We have shown that domesticated chickens are consistently less fearful and stressed than Red Junglefowl in a range of different behavioural challenge tests, and have a lower hypothalamic-pituitary–adrenal (HPA) axis reactivity (Campler et al., 2009; Ericsson et al., 2014). Furthermore, studying Red Junglefowl selected for high or low levels of fear of humans, we found that this trait was correlated to several other behavioural and morphological traits, indicating that fear may indeed play a central role in regulating a variety of domestication-related phenotypes (Agnvall et al., 2012).

In a number of experiments, we have exposed chickens to stress at different time periods during their lives (Goerlich et al., 2012; Lindqvist et al., 2007; Nätt et al., 2009). Generally, this caused permanent changes in both spatial and associative learning, as well as in foraging patterns and social dominance. Furthermore, early life stress caused a blunted HPA axis reactivity as measured by corticosterone responses to physical restraint. These phenotypic stress responses were associated with life-long changes in hypothalamic gene expression, where genes involved in neural processes and immune defence were particularly affected.

These chicken results corroborate studies by Weaver et al. in rats, showing that the corticosterone response to restraint stress was smaller in adult offspring of mothers displaying more intense maternal behaviour (Weaver et al., 2007). The epigenetic effect studied by Weaver et al. (Weaver et al., 2007) was caused by inhibition of NGFI-A binding to its consensus sequence by DNA methylation, which in turn sparked a cascade of molecular and physiological events eventually leading to the phenotypic effects.

Transgenerational effects of stress

It is clear that stress is a powerful inducer of modifications in gene expression, mediated by more or less permanent epigenetic alterations of key DNA sequences. If such alterations could be transferred to the offspring, the evolutionary implications of this should be obvious (Fig. 2).

As understood from the definition (as mentioned earlier), so-called obligatory epigenetic marks are reliably inherited together with the causative DNA polymorphism. However, facilitated and pure epigenetic modifications can also be inherited through the

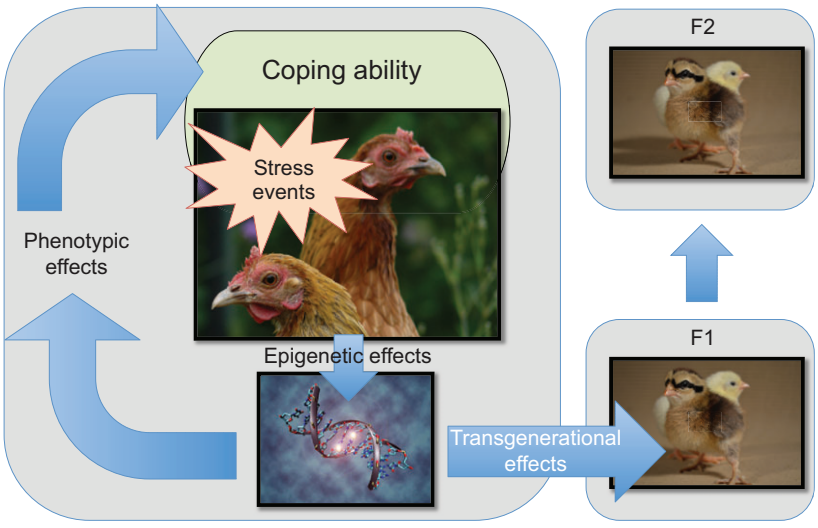

Fig. 2. Schematic representation of the interactions between stressful life events and epigenetic changes. Modifications in gene expression caused by, for example, DNA methylation may affect the later ability to cope with similar stress experiences. Furthermore, such effects may be transferred to the next generation, either directly to the F1 by, for example, *in utero* or *in ovo* hormonal effects, or to F1 and further generations by epigenetic germ-line modifications. DNA methylation figure courtesy of Wikipedia Commons, Christoph Bock.

germline in many cases, as outlined in recent reviews of extensive experimental evidence (Jablonka and Raz, 2009; Richards, 2006).

Although not stressors in the sense used in this review, toxicants are potent environmental insults, well known to cause stable transgenerational epigenetic changes following direct exposure. For example, Skinner and colleagues found that embryonic exposure of rats to vinclozilin, an endocrine disruptive and anti-androgen fungicide, caused extensive changes in the brain transcriptome, detectable for at least three generations (Skinner et al., 2008). This was associated with modifications of anxiety-related behaviour in the F3 generation of the exposed animals. In another study, the same group observed increased obesity in F3 offspring of DDT-exposed rats, which apparently was mediated by methylation changes in both sperm and eggs (Skinner et al., 2013).

In mice, early maternal separation has been found to change the DNA methylation profile in the sperm of affected males (Franklin et al., 2010). It also changed the behavioural response to aversive environments, and caused modified brain gene expression in the offspring. Similar effects have also been observed in female mice (Weiss et al., 2011).

My group has focused on chickens, which eliminates direct maternal effects, as chickens can readily be incubated and raised without parental involvement. Transgenerational effects will therefore have to be caused either by epigenetic germ-line modifications or different egg environments (e.g. maternal allocation of steroids in yolk and albumen) (Rubolini et al., 2005).

To study the extent of such transgenerational effects, Red Junglefowl and domesticated White Leghorn layers were raised under chronic stressful conditions (unpredictable light schedules), which caused both breeds to show significantly reduced spatial learning (Lindqvist et al., 2007). In White Leghorns, but not in Red Junglefowl, this was transferred to the offspring. Stressed parents of both breeds showed a modified hypothalamic gene expression profile, but this was mirrored only in offspring of the domesticated birds. Hence, the stress-induced changes in both phenotype and gene expression were transgenerationally stable in White Leghorns, whereas this was not the case in the wild ancestors. Whereas the mechanism behind this difference is not known, it has been suggested that it may have been caused by selection during domestication for increased ability to respond with epigenetic modifications (Jensen, 2014).

Can transgenerational effects also be adaptive, for example through ‘perinatal programming’, whereby females might be able to prepare the offspring for the environment into which they are born or hatched (Khulan and Drake, 2012)? This seems to be a plausible hypothesis. This would also be valid for birds, where the egg environment with respect to nutrients and hormones can be modified in response to the mother’s experiences (Henriksen et al., 2011), but the experimental evidence so far is not overwhelming.

To investigate perinatal programming, we raised domesticated chickens under chronic unpredictable light schedules (Nätt et al., 2009). As adults, birds were observed in a free-foraging test carried out in a round arena with food holes in the floor, where they could freely choose between a safe food source (fully visible standard chicken food), and a less secure but more attractive one (hidden meal-worms). Stressed birds pecked more in total, and directed more pecks to the safe food source, indicating a more conservative foraging pattern. This was mirrored in the behaviour of female, but not male, offspring. This behaviour change seems adaptive in a situation where food availability is unpredictable, as was the case in the unpredictable light schedule (as chickens do not feed in darkness). Hence, female offspring seemed to be programmed for a foraging behaviour that is adaptive in an environment with unpredictable food access. Offspring of stressed parents were also dominant in a food competition test, and had a higher growth rate and lower mortality throughout life, emphasising the apparent adaptiveness of the transgenerational effect.

There was a significant correlation of stress-induced hypothalamic gene expression across generations, showing that hundreds of genes analysed with microarrays had changed their expression similarly in parents and offspring (Nätt et al., 2009). We hypothesised that this could partly be mediated through hormones in the eggs. Indeed, there was a significant increase in oestradiol in yolk of eggs laid by stressed birds. Hence, both direct hormonal effects and germ line epigenetic effects are possible mechanisms underlying the observed transgenerational effects. Of course, the hormonal effects may also cause epigenetic changes, which adds to the complexity of the mechanisms.

Critical stress windows for transgenerational effects

Transgenerational epigenetic effects of stress appear to be ubiquitous, but it is not clear whether stress encountered during all life phases will be equally potent in causing such effects. It is quite possible that certain life phases are more susceptible. Most research has focused on the effects of early experiences. For example, in zebra finches, variation in early nutrition affects the reproductive performance of the offspring of the affected individuals (Naguib et al., 2006), so offspring fitness depends partly on the neonatal nutritional state of the parents. Several studies show that, in mammals, prenatal stress may cause both pathological and adaptive transgenerational effects (reviewed by Braastad, 1998; Kaiser and Sachser, 2005). The epigenetic mechanisms behind this remain elusive though.

We exposed chickens to stressful conditions at various time periods from hatching until sexual maturity, and examined behavioural and physiological stress responses as well as transgenerational effects on brain gene expression (Goerlich et al., 2012; M. Ericsson, R. Henriksen, A.-S. Sundman, K. Shionoya and P.J., in preparation). Early stress caused a long-term blunting of corticosterone reactivity and modifications of learning and stress-related behaviour, which was mirrored in the offspring, and there was a significant correlation of differential gene expression in the hypothalamus between the two generations. This means that, just like in the earlier studies cited above, stress-induced alterations in the gene expression profile were inherited. The gene ontology term ‘response to hormone stimulus’ was one of the most significant, relating to 10% of the differentially expressed genes.

The mechanisms of these epigenetically mediated transgenerational effects are still not known, but eggs of the stressed hens tended to be heavier and have a higher concentration of oestradiol. Hence, the offspring effects could have been caused by alterations in the egg environment. Although early stress, during the first few weeks of life, was able to create transgenerational effects, our studies indicate that the pre-pubertal period is particularly sensitive, suggesting that long-term programming of parental germ cells may in fact occur at this time (M. Ericsson, R. Henriksen, A.-S. Sundman, K. Shionoya and P.J., in preparation).

In most of our own experiments, sex differences in transgenerational effects were observed, corroborating many studies showing large sex differences in the long-term stress responses in chickens (Madison et al., 2008). This may partly be explained by genetic imprinting, the process where the expression of a particular allele is determined by its parental origin (Curley et al., 2011). For example, in humans it has been shown that sex-specific alterations

of disease susceptibility can be detected two generations after a pre-puberty starvation period (Pembrey et al., 2006).

Transgenerational effects – widening the evolutionary perspective

Although epigenetic modifications of the germ cell line have received a lot of attention, they are by no means the only way in which environmentally induced modifications of behaviour can be transferred across generations. Jablonka and colleagues (Jablonka et al., 1998), in a thought-provoking review, suggest that culture may in effect lead to similar evolutionary consequences. This refers to the fact that learnt behaviour can be copied by other individuals in a population, including the offspring of the affected individuals. The consequence of this will be that populations may differ in how individuals behave in a particular context, without this being related to genetic variation. Although mostly associated with primate behaviour (van Schaik, 2012), phenomena akin to culture have been demonstrated even in invertebrates, such as mate choice copying in *Drosophila* (Danchin et al., 2010). Hence, this may be an ancient phenomenon, with a highly underestimated importance in evolutionary processes.

Closely related to this broad idea is the observation that learnt, or otherwise environmentally modified behaviour, can act as mediator of epigenetic transgenerational effects. Such behaviour may be able to reinstate a particular epigenetic modification in the next generation. For example, as shown in the previously cited study by Meaney (Meaney, 2001), female rats that had received more licking and grooming behaved in a similar way when they themselves became mothers, showing more arched-back nursing and grooming. As this behaviour seems to be instrumental in creating the epigenetic mark necessary for its own propagation, possibly related to methylation patterns affecting the expression of the oxytocin receptor gene, the transgenerational effects may therefore rely on a reinstatement of both the phenotype and the epigenotype in every generation. This aspect of transgenerational epigenetic inheritance of behaviour variation has as yet not received much research interest, but may be a key aspect of adaptation and evolution.

It is a well-established opinion that evolutionary change relies on random mutations in DNA sequence (Futuyma, 1997). Hence, the only important genetic factor to take into account when considering the evolutionary development of populations would be DNA polymorphisms. However, as seen in this and in several other reviews on epigenetics, the genome is able to respond dynamically to events in the environment, sometimes causing consistent alterations in the orchestration of gene expression, with these changes sometimes being transgenerational (Jablonka, 2013). This calls into question some fundamental corner-stones of 20th century evolutionary theory, as it implies that heritable responses to the environment can modify phenotypes and should therefore be considered central to evolution itself. Hence, we need to consider not only DNA polymorphisms as the source of heritable genetic variation. As epigenetic variants can affect phenotypes and be inherited in much the same manner as alleles, they should also be included when we account for the total variation available for selection (Heyn et al., 2013). One way forward is to map the epigenetic variation and the genetic causes of it, using methylation QTL analysis (Gibbs et al., 2010). The results of such analyses will allow us to understand the extent to which epigenetic variation depends on DNA polymorphisms. The second step would be to use similar tools to map the connection between methylation differences and phenotypic variation, and such studies are currently being conducted in my group, focusing on chickens.

Another evolutionary implication of recent discoveries is the possibility that selection may act not only on the existing epigenetic variants but also on the ability to respond epigenetically to environmental challenges. The logic behind this would be that an individual that is able to respond to stresses of various sorts by altering the epigenome in a transgenerationally stable way would gain a selective advantage. Although this is as yet a highly speculative suggestion, some of our own data may actually be understood in this light. As described earlier, domesticated White Leghorn chickens responded to stress with transgenerational changes in gene expression and associated behaviour modifications, but ancestral Red Junglefowl did not (Lindqvist et al., 2007). Possibly, this could be the result of selection during domestication for individuals that are able to respond epigenetically to the stress associated with living in close proximity to humans. This is to some extent supported by the fact that, in a comparison of hypothalamic gene expression and methylation between domesticated and wild chickens, domesticates were hyper-methylated on more than 70% of the differentially methylated loci, indicating that extensive methylation has been acquired during domestication (Nätt et al., 2012).

As mentioned above, there is as yet limited data to support the evolutionary importance of epigenetic variation. But one exciting experiment shows the potential of the phenomenon. Cropley and co-authors (Cropley et al., 2012) used the pseudo-agouti phenotype in mice as a model. This colour morph is caused by a methylated epi-allele. When selecting for the phenotype in an environment with methyl-donor enriched food, the frequency of the phenotype increased over the five generations included in the experiment. In a control group, kept on non-supplemented food, this response to selection was not observed. This strongly suggests that epigenetic variation can indeed be a substrate for natural selection, where adaptive phenotypes can evolve.

Lastly, an exciting possibility is that epigenetic variation may be related to DNA mutations. This is a highly speculative suggestion, but two different possible mechanisms may perhaps mediate such a phenomenon. Firstly, methylation of genes is usually associated with transcriptional silencing, which would make the specific gene more accessible for mutation as the DNA repair mechanisms would not act as efficiently. Secondly, methylated CpG-nucleotides are known to be more likely to mutate, which causes a non-random mutation rate that is linked to epigenetic variation (Tsunoyama et al., 2001). Both of these mechanisms would cause mutations to be non-random, even perhaps responsive to environmental insults. If this can be demonstrated with experimental testing, it would call for a thorough revision of current evolutionary theory.

Conclusions

Animal behaviour is largely under the control by genetic mechanisms, governing sensory abilities, endocrine responses and neural mechanisms. Whereas it has long been assumed that phenotypic variation is caused solely by genetic polymorphisms interacting with the environment, it is now clear that environmental input also modifies the orchestration of the genome and thereby causes long-term effects on behaviour. This is mediated by, for example, stress-induced alterations of DNA methylation and histone modifications, which subsequently alter gene expression. Evidence from several species now suggests that stress in different life phases can cause phenotypically potent epigenetic changes, which may be transferred to the offspring and even be maintained for several generations. Domestication provides a helpful and important model system for studying the evolutionary implications of epigenetic variation. It has been found that domestic animals differ widely from

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their wild ancestors with respect to brain gene expression, and in chickens, a massive increase in DNA methylation has accompanied its domestication. This highlights the potential importance of epigenetic variation in behavioural evolution.

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