Molecular identification of colour pattern genes in birds

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Molecular identification of colour pattern genes in birds

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Dedication

Für meine Familie und alles was ihr für mich getan habt.

Familie ist wie ein Baum.
Die Zweige mögen in unterschiedliche Richtungen wachsen.
Doch die Wurzeln halten alles zusammen.

Autor unbekannt

'It's an awful stretcher to believe that the peacock's tail was thus formed but…most people just don't get it – I must be a very bad explainer.'
Charles Darwin

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Schwochow Thalmann D**, Bornelöv S, Li J, Dorshorst B, Bed'Hom B, Gourichon D, Tixier-Boichard M and Andersson L. Autosomal barring in the domestic chicken is strongly associated with segregation at the *MC1R* locus. Manuscript.

II **Schwochow Thalmann D**, Ring H, Sundström E, Cao X, Larsson M, Kerje S, Höglund A, Fogelholm J, Wright D, Jemth P, Hallböök F, Bed'Hom B, Dorshorst B, Tixier-Boichard M and Andersson L. (2017). The evolution of Sex-linked barring in chickens involves both regulatory and coding changes in *CDKN2A*. PLoS Genetics 13 (4).

III Lamichhaney S, Fan G, Widemo F, Gunnarsson U, **Schwochow Thalmann D**, Hoeppner PM, Kerje S, Gustafson U, Shi C, Zhang H, Chen W, Liang X, Huang L, Wang J, Liang E, Wu Q, Lee M-YS, Xu X, Höglund J, Liu X and Andersson L. (2016). Structural changes underlie alternative reproductive strategies in the ruff (*Philomachus pugnax*). Nature Genetics 48 (1), 84-88.

IV **Schwochow Thalmann D**, Mandrika I, Roga A, Fridmanis D, Lank DB and Andersson L. Functional characterization of an *MC1R* allele associated with white colour in the satellite male ruff (*Philomachus pugnax*). Manuscript.

Other Work by the Author

(Not included in the thesis)

Imsland F, McGowan K, Rubin C-J, Henegar C, Sundström E, Berglund J, **Schwochow D**, Gustafson U, Imsland P, Lindblad-Toh K, Lindgren G, Mikko S, Millon L, Wade C, Schubert M, Orlando L, Penedo MCT, Barsh GS, Andersson L (2016). Regulatory mutations in *TBX3* disrupt asymmetric hair pigmentation underlying Dun camouflage colour in horses. Nature Genetics 48 (2), 152-158.

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Abbreviations

αMSH	α-melanocyte stimulating hormone
3'UTR	3' untranslated region of mRNA
5'UTR	5' untranslated region of mRNA
ARF	alternate reading frame protein
ARF-BP1	ARF-binding protein 1
ASIP	agouti signalling protein
ATP	adenosine triphosphate
B	*Sex-linked barring* locus in chicken
BAX	BCL2 associated X protein
BCDO2	beta-carotene dioxygenase 2
bp	base pairs
BrdU	bromodeoxyuridine
cAMP	cyclic adenosine monophosphate
CDH1	cadherin 1
CDKN2A	*cyclin dependent kinase inhibitor 2A*
CENP-N	centromere protein N
Co	*Columbian* locus in chicken
CREB	cAMP responsive element binding protein
CYB5B	cytochrome B5 type B
Db	*Dark brown* locus in chicken
EDNRB2	endothelin receptor B subtype 2
FACS	fluorescence-activated cell sorting
F_{ST}	Fixation index
GAS8	growth arrest specific 8
Gb	giga bases
HEK 293	human embryonic kidney cells 293
HSD17B2	Hydroxysteroid 17-beta dehydrogenase 2
IBD	identical-by-decent

Kb	kilo bases
MART1	melanoma antigen recognized by T-cells, melan-A
Mb	mega bases
MC1R	melanocortin 1 receptor
MC2R	melanocortin 2 receptor
MDM2	mouse double minute 2 homolog
MITF	microphtalmia-associated transcription factor
Ml	*Melanotic* locus in chicken
Mo	*Mottling* locus in chicken
MRAP	melanocortin 2 receptor accessory protein
MRAP2	melanocortin 2 receptor accessory protein 2
NGS	next generation sequencing
NQO1	NAD(P)H quinone dehydrogenase 1
$p21^{CIP}$	cyclin dependent kinase inhibitor 1A
p53	tumor protein p53
Pg	*Patterning* locus in chicken
PHLDA3	pleckstrin homology like domain family A member 3
PKA	protein kinase A
qPCR	quantitative polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RNA-seq	RNA sequencing
SDR42E1	short chain dehydrogenase/reductase family 42A, member 1
SNP	single nucleotide polymorphism
SOX10	SYR-related HMG-box 10
TRP1	tyrosinase related protein 1
TRP2/DCT	tyrosinase related protein 2
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
TYR	tyrosinase
VAI	Variant Annotation Integrator
WWP1	ww domain containing E3 ubiquitin protein ligase
YWHAB	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein beta

1 Introduction

1.1 Pigment patterning in birds

Plumage pigmentation diversity in birds is without comparison. Not just the range of displayed colours is impressive but also the patterning across the avian body and on individual feathers. In contrast and with only few exceptions e.g. in the order primates, mammals rarely display vibrant colours. Mammalian pigment patterning mainly occurs in form of patches, spots and stripes across the body and can be found e.g. in the genus *Equus* and *Rodentia*, in the family Felidea as well as in a broad range of domesticated animals such as pigs, horses, dogs and cats. Pigment deposition can also vary within a single mammalian hair and alter the overall appearance of the fur (Manceau *et al.*, 2011, Imsland *et al.*, 2016). Compared with opportunities provided by the two dimensional patterning possibilities on pennaceous bird feathers, the essentially one-dimensional patterning of animal hair is much less complex and cannot possibly give rise to the patterning variation observed in many extant bird species. It appears plausible that the genetic basis underlying pigment pattern formation in birds most likely is more complex than in mammals and, unfortunately, this field of research is poorly developed.

1.1.1 Pigment and colouration forms in birds

Plumage colouration in birds can be created by several mechanisms. It can be the result of substances named pigments, which absorb a certain wavelength of light or is created through light scattering as a result of specific structures of or in feathers (structural colours) (Lucas and Stettenheim, 1972, Fox, 1976, Prum *et al.*, 1999). In many feathers a combination of both mechanisms is common. In birds, three pigment type groups are known to contribute to plumage variation: carotenoids, melanin and uncommon colours such as porphyrins.

Carotenoids are the second most prevalent pigment in the avian integument and have been shown to be responsible for red, orange and yellow colouration of skin, scales, eggs in fishes, amphibians and reptiles, feathers, beaks, facial wattles, combs, eyes and tarsal skin. The only integument that has not been found to be coloured by carotenoids is the mammalian hair (Hill and McGraw, 2006). The orange hue of the goldfish (*Carassius auratus*), the yellow colour of the common canary (*Serinus canaria*) as well as the deep pink of the greater flamingo (*Phoenicopterus ruber*) can all be attributed to carotenoids. However, there are other pigments, which are able to confer a similar set of colours such as the psittacofulvine in parrots (referred to in more detail below). In 1934, Brockmann and Völker were able to prove that birds are unable to synthesize carotenoids themselves by experimentally feeding a carotenoid deprived diet to canaries during moult (Brockmann and Völker, 1934). The birds grew white feathers, instead of the typical yellow. Although animals lack the enzymes to manufacture carotenoids from the common precursor, they are able to metabolize carotenoids into different forms (Brush, 1990), which are present in plumage, skin legs and beak but not in their original consumed diet. Today carotenoid colouration has been described in over 150 bird species spanning several different orders with much more being presumed but not proven (Hill and McGraw, 2006).

The most common pigment in animals and birds is melanin (Hill and McGraw, 2006). All birds, except those exhibiting the albino phenotype, have some melanin pigment in some body parts. Melanins are responsible for black, brown, grey, rufous, chestnut and buff shades in plants, fungi and animals. Even the brown, damaged skin on fruits is the result of melanisation. Two main different types of melanin have been described: eumelanin and pheomelanin. Eumelanin is believed to be the larger form of the two melanins, which is responsible for dark black or brown hues in invertebrates and vertebrates alike. It is stored as granules in oval melanosomes and is insoluble in nearly all solvents. Pheomelanin is responsible for the reddish-brown pigments and predominates e.g. in human red hair, red and yellow fur of mammals as well as in chestnut and rufous feathers. Its lower molecular weight and presence in smaller, globular granules, which are soluble in alkaline solutions, suggests that pheomelanin has quite different structural and light absorbance characteristics than eumelanin. Melanins are not derived from the diet as carotenoids but are produced endogenously in peripheral tissue like skin, more specifically in the specialized cells called melanocytes present in the epidermis of birds and mammals (Mason and Mason, 2000). Melanocytes transfer pigment to keratinocytes, cells that later become keratinized and die. This type of fixed, morphological colouration of hair, feathers, beaks and scales is in

stark contrast to the much more flexible pigmentation strategy in poikilothermic vertebrates that use layered pigment systems of various chromatophores enabling them to spontaneously blend in with a changing environment (Bagnara and Hadley, 1973). Melanin-based colouring, however, is not static either. It comes in a variety of seasonal, sexual and integumentary forms. There are four classes of hormones, which are known to affect melanin pigment production including androgens, estrogens, pituitary hormones (e.g. luteinizing hormone) and thyroid hormones (e.g. thyroxin). Melanin-based pigmentation provides the ultimate base for a colourful breeding plumage observed in many bird species, and may serve as a potential honesty-reinforcing mechanism, as it is maintained by hormonal effects with potential consequences on metabolism and immune (Folstad and Karter, 1992). A collection of melanin profiles in 13 different bird species spanning four orders suggests that all melanin-containing feathers analysed harbour both eu- and pheomelanin, albeit occasionally in very low amounts (e.g. 0.8% eumelanin in rufous cheek patches of male zebra finches). The analysis further revealed that the total concentration of melanins is less meaningful in shaping variability than is the relative proportion of the two pigment types. It was also found that melanins in feathers could simultaneously occur with other pigments (such as carotenoids). To account for this 'melanin mixture' observed in all feathers, it was therefore suggested to introduce labels such as 'eumelanin dominated' or 'pheomelanin-dominated' feathers (Hill and McGraw, 2006).

Even though no black pigment other than eumelanin has been identified until today, brown, chestnut, grey and buff hues can also be the result of the presence of other, more rare pigments (McGraw *et al.*, 2004). Porphyrin pigments as an example, give the brown or rufous colour to egg shells and feathers of bustards and owls (With, 1978, With, 1974). Carotenoids and pterins confer yellowish and orangish colours. Melanins can mask or alter the presence of other brightly coloured pigments (e.g. turacin in turacos). Porphyrins are produced in liver and peripheral tissues such as the oviduct and colour the irises of many birds like raptors, pigeons, blackbirds and starlings in vibrant orange, yellow and white (McGraw *et al.*, 2004). Carotenoids and melanins are less likely to be part of eye colouration than pterins. Psittacofulvins produce an impressive set of brilliant colours limited to a single order of birds: the parrots (Hill and McGraw, 2006). Psittacofulvins cannot be found in the blood or liver, which implies that they are produced directly in feathers (McGraw and Nogare, 2004).

Birds can also display colours due to interference with microscopically small structures within the feather. Those termed structural colours often occur together with other pigments such as melanins to create an overwhelming

amount of vibrant colours. The eye in the peacock tail feather is probably the most impressive example of structural colours combined with melanins (Hill and McGraw, 2006). Parrot colouration can also be the result of a combination of structural and pigment colour. As an example, green plumage in e.g. budgerigars is the result of a combination of yellow psittacofulvin and blue structural colouration. The appearance of the green in parrots is quite different from the green resulting from a combination of carotenoids and melanins as observed e.g. in European greenfinches (*Carduelis chloris*) (Lucas and Stettenheim, 1972).

The great variation of pigments in birds is overwhelming and has not been studied well in terms of pigment production and molecular processes of pigment distribution. In recent years methodological advances in whole genome sequencing made it possible to identify a number of genes involved in pigment production and distribution. Most notably the first three enzymes involve in carotenoid and psittacofulvin syntheses respectively could be pinpointed. In chicken a differential expressional regulation of the enzyme beta-carotene dioxygenase 2 (BCDO2) in skin has been shown to cause the yellow skin phenotype (Eriksson *et al.*, 2008). Similarly in canary birds a new enzyme was described, cytochrome P450/CYP2J19, which is up-regulated in skin and feathers of red canary birds and is predicted to function as a ketolase to mediate the red colouration (Lopes *et al.*, 2016). In budgerigars, Cooke *et al.* identified a missense mutation, R644W, in a so far uncharacterized polyketide synthase as the causative variant for a lack of yellow colour in parakeets (Cooke *et al.*, 2017). They found that the mutation is abolishing the enzymes function and that regulatory changes affecting this enzyme play a major role in colour establishment in this bird order.

1.1.2 Function of pigmentation in birds

The impressive diversity of pigments identified in bird feathers up to date are predicted to serve numerous functions such as protection from various destructive sources, aiding in thermoregulation, facilitating camouflage and communication between and within species.

In mammals the protection from UV light by melanin pigment in the skin is a widely studied and accepted phenomenon. In birds however, melanisation has rather been studied in feathers than skin. As a consequence, the role of melanins in UV protection in bird skin is largely unexplored (Hill and McGraw, 2006). What has been studied is the contribution of pigments, in particular melanins, in mechanical protection of feathers. Like all polymers melanins are assumed to contribute to the hardness of biological tissue (Moses

et al., 2006, Bonser, 1995) with no apparent differences between the different melanin forms (Pannkuk *et al.*, 2009). Flight feathers in many species often exhibit melanised and non-melanised areas and research has shown that those melanised parts have a greater ability to resist mechanical deformation compared to the non-melanised areas (Bonser, 1995). Apart from their mechanical properties, melanins are also proposed as being a primitive part of the innate immune defence system (Mackintosh, 2001) and protect the feathers they colour from feather-degrading bacteria (Burtt and Ichida, 2004). The evidence for this, however, is conflicting, since feathers themselves serve as substrate for some bacteria (Grande *et al.*, 2004, Gunderson *et al.*, 2008) and do not appear to protect against feather degrading lice (Bush *et al.*, 2006).

Melanised areas in ectotherms are known to increase their temperature more than non-melanised structures, a process that has not been well studied in birds (Hill and McGraw, 2006). When outside temperatures are low, maintaining body temperatures in endotherms such as birds might become important (Bech and Praesteng, 2004) and dark feather pigment can aid in this process (Margalida *et al.*, 2008). It needs to be taken into consideration, however, that dark feather pigmentation also occurs in regions with higher ambient temperatures and that those birds do not show any sign of heat compensatory mechanisms, such as vascularized parts of bare skin (Hill and McGraw, 2006). Specific thermo-regulatory properties of different melanins have not yet been investigated. In the mainly eumelanin-containing, grey morphs of tawny owl (*Strix aluco*), dorsal feathers have larger and denser plumulaceous parts compared to the brown morphs (Koskenpato *et al.*, 2016). Moreover, melanic tawny owls appear to have a better survival rate in cold winters. This could suggest that melanin can contribute to improved thermoregulation of these plumulaceaous feather parts. (Galvan and Solano, 2016) proposed another hypothesis: that the pigment form is advertising individual quality through the consumption of cysteine during pheomelanin production, which under thermal stress is increasing the amount of reactive oxygen species and stress for the organism. Thermoregulatory abilities of other pigments are less well studied, however, in an early study, porphyrins in eggshells were found to not absorb infrared light and do most likely protect eggs in warmer climates from overheating (Bakken *et al.*, 1978).

Pigmentation is predicted to confer crypsis, either to avoid being predated on or to avoid being detected by prey (Hill and McGraw, 2006). A particular interesting combination of both camouflage and mimicry was developed in the parasitic common cuckoo (*Cuculus canorus*) as a consequence of host-parasite coevolution. The melanic form in this bird species was proposed to mimic the Eurasian sparrow hawk (*Accipiter nisus*) (Davies and Welbergen, 2008, Davies

and Welbergen, 2009) while the rufous morph is more resembling the Eurasian kestrel (*Falco tinnunculus*) (Voipio, 1953, Trnka and Grim, 2013). The barred feather pattern in both morphs aids the birds to blend into the environment when the female is waiting in the vicinity of the host nest, while on the other hand their resemblance to birds of prey reduces the aggression of the host when the cuckoo female is approaching the nest for laying her eggs (Gluckman and Mundy, 2013).

The function of barred plumage is debated, with evidence supporting two main themes: camouflage and sexual communication/ sexual selection. A recent survey of over 90% of extant bird species revealed that barred plumage seems to occur in a higher frequency in species with a strong sexual dimorphism towards barred plumage in females, suggesting that the main function of barring is camouflage (Gluckman and Cardoso, 2010). Indeed, plumage with bars is more difficult to track on plain surfaces (Bradbury and Vehrencamp, 1998, Cuthill *et al.*, 2005, Stevens *et al.*, 2008) but through its regular pattern formation within and between adjacent feathers can function as an amplifier signal. Disruption of the regular pattern is easily recognizable, signalling badly maintained or damaged plumage resulting e.g. from aggressive conflicts with conspecifics. However, the authors also note that compared to other plumage pattern such as mottling, barring is more frequently biased towards adult and male birds. This highlights that in many species barring is most likely also maintained as a sexual communication signal (Gluckman and Cardoso, 2010). Furthermore, another recent survey encompassing 80% of all bird species did not reveal convincing evidence that plumage pattern are associated with a particular habitat, at least on a global and taxonomic scale (Somveille *et al.*, 2016). Species with both regular and irregular plumage pattern appear to be distributed randomly across the globe providing evidence that camouflage is not a major purpose of pigment pattern but rather signalling.

Other pigments can also be involved in sexual signalling. Burrowing parrots (*Cyanoliseus patagonus*) carry patches of red feathers on their belly and pairs have been shown to mate based on the size of the patch; a confirmed signal of individual quality (Masello and Quillfeldt, 2003). The red colour in the burrowing parrot is the result of psittacofulvin but very little is known about its production pathway and the metabolic investment associated with its production. Like natural porphyrins (Afonso *et al.*, 1999, McGraw, 2005), psittacofulvine can exhibit antioxidant activity (Morelli *et al.*, 2003), which might affect individual quality and can be signalled through the pigment itself. In contrast, much more is known about the molecular basis of melanin production and a number of hypotheses have been postulated about how this pathway can signal individual quality. Melanin-based pigments are considered

an honest signal of quality, as birds with larger or more intense colour patches are preferred for mating and therefore possess a higher fitness (McGraw, 2008, Guindre-Parker and Love, 2014). Melanogenesis branches off in two pathways depending on whether eumelanin or pheomelanin will be produced (further discussed below). Pheomelanin production requires cysteine, an antioxidant, which can be limited under environmental challenging condition, as it is required elsewhere in the body. It has therefore been hypothesised that pheomelanic traits are particular costly (Galvan and Alonso-Alvarez, 2009, Galvan and Solano, 2009) and represent a more reliable sign of individual quality than eumelanic traits. This concept might be supported by the fact that relatively few bird species exhibit a pure pheomelanic plumage (Galvan and Solano, 2016). Eumelanized feathers only appear to posses a true signalling character through melanic pattern formations such as barring as described above.

1.1.3 The bird feather

The ultimate location of pigment pattern formation in birds is the feather. Feathers are skin appendages that define the Aves class. They are considered the most complex appendages found among vertebrates, which function in thermoregulation, communication and flight (Chatterjee, 1997, Chiappe, 1995). Feathers come in different forms, sizes, and colours and depending on the species can reach between 20,000 to 80,000 feathers per bird. Feathers comprise five important developmental and structural features (Chuong *et al.*, 2003):

1. Feathers possess localised zones of proliferating cells positioned proximally within a proximal-distal growth mode.
2. While growing, the feather is created by hierarchical levels through the formation of branches of the rachis, barbs and barbules (Figure 1).
3. The feather develops within a feather follicle structure and
4. When mature the two sides of the feather vane face the previous basal supra-basal layer. The pulp is no longer present.
5. The feather follicle contains a dermal papilla and stem cells, which provide the bird with the possibility to go through regeneration cycles like moulting and generating after plucking (Figure 2).

A bird can carry up to four different types of feathers, which usually differ in structure and pigmentation and serve different purposes: downy feathers, contour feathers, tail feathers (retrices) and wing feathers (remiges) (Lucas and Stettenheim, 1972). Feathers are already produced during embryonic

development (natal and juvenal feathers) and exhibit a different structure compared to the first basic plumage developing while the chick is maturing into an adult bird (Humphrey and Parkes, 1959).

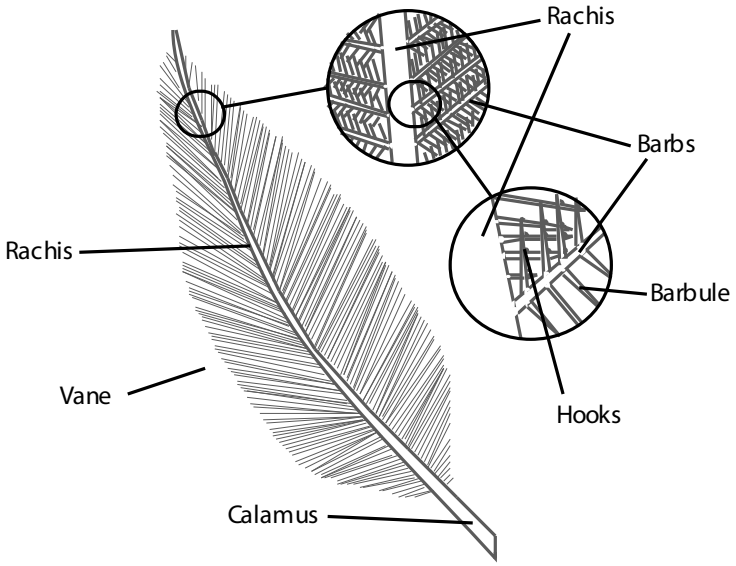

Figure 1. Anatomy of a pennaceous feather. The feather is attached to the feather follicle by the calamus. Barbs branch into smaller barbules, which interlock through tiny hooklets. This feature provides not just the rigid feather surface exterior suitable for flight but also a planar surface to exhibit within-feather pigment pattern.

If a feather is lost, by force or in the process of moulting, the feather follicle will usually generate a new feather within up to 14 days. Moulting cycles are divided into a growth and resting phase (Figure 2; (Lucas and Stettenheim, 1972)). The growth phase typically starts as soon as the old feather is detaching from the follicle and the follicle is bringing about a new feather showing red pulp with blood vessels in the growing feather shaft. The feather keeps on growing for days or months depending on its final length. When the feather has reached is final size, it starts to fully open the feather vanes and the pulp starts to degenerate. The feather becomes a dead and hollow skin appendage. The feather remains attached to the feather follicle through the shaft. The resting phase can last between two days and up to 14 months until the feather shaft sloughs off through cell differentiation (Wu *et al.*, 2004). Most birds typically moult twice a year: once in spring to generate a plumage associated with reproduction, and again in fall for a plumage with more protective purpose. The entire process is very well orchestrated in order to avoid a functional disruption of the plumage. It is an important part of the great pigmentation

diversity observed in birds, that the same feather follicle is not programmed to produce the same feather in shape, size or colour (Wu *et al.*, 2004). For example downy feathers can precede flight feathers, or sex hormones in the peacock transform plain brown feathers into its famous colourful tail feathers. Therefore every moulting event gives the bird a chance to remodel its feathers in response to an altered environment (Wu *et al.*, 2004).

Initially it was thought that feathers were unique to avian lineage but emerging fossil evidence has shown that primitive forms of feathers have existed long before the first birds emerged. Non-avian dinosaurs, which were present already about 200 million years ago in the Mesozoic era, carried an abundance of different feather-like skin appendages (Feduccia, 1999, Chiappe, 1995). Feathers have evolved from reptile scales and two different mechanisms have been proposed for their detailed morphogenesis (Wu *et al.*, 2004). Recent studies did show that the earliest feather most likely was a single, tubular filament, which eventually diversified by producing barbs (Xu and Guo, 2009). The branched structures imply that the most likely initial purpose of feathers was thermoregulation while their suitability for flight evolved much later (Norell *et al.*, 2002). Fossils suggest that 'proto-feathers' were already present in early dinosaurs such as theropods. (Li *et al.,* 2010) were able to almost fully reconstruct the colour patterning of a fossil of *Anchiornis huxleyi*. They investigated size, shape, density and distribution of the pigment-containing particles and compared them to modern bird samples. *A. huxleyi* most likely had black and grey body plumage, its head was grey and mottled with rufous and black. Its wing feathers were similarly pigmented to modern birds. This may indicate that, from very early on, melanin-based pigmentation was serving as an important mechanism facilitating feather variation, but also that selection for its signalling function may have been important in the early evolution of feathers (Li *et al.*, 2010). The common ancestor of Aves and Anchiornis had the capacity to develop white, grey, black and rufous plumage colour patterning. Within-feather pigmentation, such as spots and stripes, however, appeared later and coincides with origin of more complex, elongated pennaceous feather structure in the most common ancestor of Manirapotora (Li *et al.*, 2010).

1.1.4 The feather follicle

The feather follicle describes an epidermal structure invaginated into the dermis, surrounding and giving rise to the feather cylinder (Yu *et al.*, 2004). It is formed during early embryonic development in very complex, carefully orchestrated processes (Yu *et al.*, 2004). Structurally, they are very similar to

hair follicles but have developed through convergent evolution (Wu *et al.*, 2004). Feather follicles are connected with each other and other body parts through muscles, nerves, blood cells and connective tissue. To a bird they are a unique and sensory organ essential for flight (Yu *et al.*, 2004).

During the growth phase, barb ridges will form from a feather filament through epithelial in- and evagination. The barb ridges will differentiate into barb plates, axial and marginal plate. The keratinized barbs will arise from the barb plate while marginal and axial plate will undergo apoptosis to become spaces (Chang *et al.*, 2004). Barbs and barbules continue to differentiate to form their characteristic, complex structure. The central pulp will also degrade its own cells by programmed apoptosis to allow the feather to unfold and take on its characteristic shape. In contrast to the 'branching morphogenesis' where lung and mammary gland formation occurs through differential proliferation of growing bud tips, the morphogenesis of the feather is named 'reverse branching morphogenesis' due to the formation from the follicle (Wu *et al.*, 2004). Feathers are keratinized during their morphogenesis with two different types of keratin, α- and β-keratin (Sawyer *et al.*, 2000).

In a feather follicle with a fully-grown feather, melanocyte progenitor cells are present in a 3D ring in the papilla ectoderm at the base of the feather follicle (Lin *et al.*, 2013) (Figure 2). This stage is consistent with a quiescent state and termed the 'resting phase'. If the feather is lost or plucked accidentally, the follicle will start to generate a new feather. Melanocyte progenitor cells will migrate up the newly grown feather shaft and both proliferate and ultimately differentiate into mature, pigment-producing melanocyte (Figure 2). Melanocyte progenitor cells are initially unpigmented and negative for differentiation markers associated with melanin production (Lin *et al.*, 2013). From the middle bulge upwards they progressively become positive for typical melanocyte markers such as microphtalmia-associated transcription factor (MITF), melanoma antigen recogniced by T-cells (MART-1), tyrosinase associated protein 1 (TRP1) and tyrosinase (TYR). They increase in size and dendricity and numbers of melanin particles (Lin *et al.*, 2013). There may be between one and four melanocytes in each barb ridge (Yu *et al.*, 2004). The cycling of melanocytes into the barb region of the feather follicle appears to happen much quicker and more actively than in mammalian hair (Lin *et al.*, 2013).

In 2013, Lin *et al*., proposed a generic model highlighting four dimensions facilitating within-feather patterning:

1. Pigment can be distributed in a spatio-temporal manner along a proximal-distal axis of the feather follicle.

2. When the feather vane opens a novel medial-lateral dimension is created, providing more opportunities for displaying patterning.
3. Interactions between the heterogeneous cell populations within the follicle e.g. the cells in the pulp can affect melanocyte maturation and ultimately pigment production.
4. Other physiological or environmental factors such as hormones and seasons can alter pigmentation and morphology of the feather.

The last dimension highlights an important aspect of within-body patterning as it provides the opportunity for flexible regional cues to each individual feather follicle driving different colouration of different body parts.

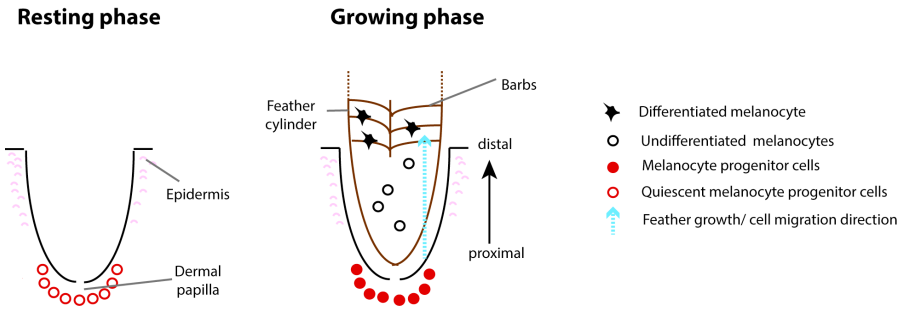

Figure 2. Anatomy of the feather follicle during resting and growing phase. When the feather in the follicle is fully grown, quiescent melanocyte progenitor cells are present at the base of the feather. If the feather is plucked or lost through moulting, the melanocyte progenitor cells become activated, migrate up the growing feather shaft, proliferate and differentiate into pigment-producing melanocytes.

1.1.5 Theory of natural pattern formation

Self-regulated pattern formations around us are omnipresent. They can reveal themselves to us in the physical world in forms of sand dunes and layers in rocks, or on different levels in living organisms from the organisation of tissues or organs, to interactions between cells or enzymes and molecules. Scientists in various fields have been intrigued by natural pattern formations and have tried to understand these complex systems. Pattern formations, irrespectively of their actual nature, can be modelled mathematically. Alan Turing, the renewed English computer scientist and mathematician, developed one of the most profound models to explain biological pattern formation named the Turing or the Reaction-Diffusion model (Turing, 1952). Turing proposed two 'morphogens', 'two diffusible substances interacting with each other'. One of the morphogens would work as an 'activator', while the other one is an

'inhibitor'. The activator is both activating its own as well as the production of the inhibitor. Turing predicted that if the amount of both morphogens starts increasing, their ranges are expanding too. If the inhibitor range were expanding faster than those of the activator, a pool of activators would eventually be surrounded by a pool of inhibitors, creating a circular pattern. The interaction between both morphogens makes the system self-regulating and as a consequence provides it with the ability to generate various patterns independent of the pre-pattern (Kondo and Miura, 2010).

How applicable is the mathematical theory of the Reaction-Diffusion model to pattern formation observed in natural systems? Turing's model predicts e.g. that pigment pattern formation on bigger bodies like the torso leads to production of spots, while smaller body parts such as legs and tails should rather exhibit stripes. This behaviour is observed e.g. in fur pigmentation of felids. The Reaction-Diffusion model has also been successfully applied to the involution of sea shells (Meinhardt, 1995). In 2002, Prum *et al.*, altered various parameters in Turing's model and were able to create a number of naturally observed within-feather pattern such as lacing, barring and even the eye on the peacock tail feather as well as pattern transitions observed along the body of birds. The author's findings strongly predict that within-feather pattern formation is determined by antagonistic interactions among molecular expression gradients within the feather follicle (Prum and Williamson, 2002). The precise identity of processes and components involved in such interactions remains largely unknown up to date. It also remains to be elucidated on which hierarchical level these processes and components act. Is it the absence or presence of melanocytes and/or melanocyte progenitor cells that is ultimately responsible for within-feather patterning? Or does the patterning happening on the level of melanosome formation, activity and transfer to keratinocytes?

1.2 Melanin-based pigmentation

Birds are popular for their flamboyant, vibrant colours but it actually appears to be the rather monotonous melanin pigments that form the greatest variation: such as bars, stripes and dots on individual feathers; patterns, which are generally not generated by other colour pigments. Out of 9049 extant bird species, approximately 32% exhibit a complex pattern and the overwhelming majority of these (98%) are the result of melanin-based colours (Galvan *et al.*, 2017). Only 53 species spanning 3 families represent an exception from the melanin rule, which may be due to rare innovations in the carotenoid metabolism (Prum *et al.*, 2012) and not structural colours (Maia *et al.*, 2011). Structural colours arise as a combination of microscopic structures in the

feather as well as melanins that absorb light at certain wavelengths (D'Alba *et al.*, 2012), suggesting that melanin is also contributing to structural colour pattern formation. Assuming that pigment patterning has primarily evolved for signalling (Somveille *et al.*, 2016), the observed diversity might be better explained by the process of pigment production itself as it is associated with physical constraints and provides a base for natural selection to act on (Galvan and Solano, 2009).

1.2.1 The melanocyte

The term melanocyte describes a type of cells, which is specialized in producing melanin pigments in mammals and birds. Many fish, amphibians and reptiles are also capable of producing melanin. The defensive black ink seen in cephalopods is probably one of the most visual examples. However, melanins in these groups are produced by a slightly different type of cell named melanophore. Melanocytes are most common in the skin, hair and feather follicles as well as in the eye (Pascal *et al.*, 1997), but have also been found in the inner ear, oesophagus, thyroid, bones, heart and even the brain (neuromelanin; e.g. (Zecca *et al.*, 2003)). It was suggested that melanocytes are the only pigment-producing cell in birds and mammals (Hach *et al.*, 1993) but Hill and McGraw, 2006, pointed out that e.g. the biological entities producing colour to the avian eye have yet to be described. Melanocytes develop from melanoblasts (Bagnara *et al.*, 1979), which are derived from neural crest cells (Bagnara *et al.*, 1979). During the first few days of the embryonic period in birds, melanoblasts migrate into the skin and get positioned in the middle of the epidermis of the developing feather germ (Strong, 1902, Greite, 1934, Watterson, 1942, Rawles, 1944). Towards the end of the first week, the melanoblasts start to differentiate and develop cytoplasmatic processes, which eventually deliver melanosomes filled with melanin particles to keratinocytes that give rise to the feather filaments (Yu *et al.*, 2004). Here, the melanosomes are taken up by the keratinocytes by phagocytosis (Lucas and Stettenheim, 1972, Greite, 1934, Watterson, 1942, Jimbow and Sugiyama, 1998).

1.2.2 Melanogenesis

The production of melanins is a complex process, which has not been illuminated to its full extent yet. It is not a unique or unaltered biosynthetic pathway as animals, plants and microorganisms show some differences in the nature of precursors and enzymatic machinery (Solano, 2014). In mammals and birds, melanin is produced in small organelles named melanosomes, which

contain all the enzymes required for the process (Hearing, 2000, Sulaimon and Kitchell, 2003). Avian melanins are formed from the aromatic amino acid tyrosine (Lerner *et al.*, 1950) (Figure 3), which is an amino acid that must be acquired through diet or synthesised using phenylalanine. The enzyme TYR catalyses the initial oxidation step of tyrosine to dopquinone. TYR is a highly conserved enzyme across mayor animal lineages (Sato *et al.*, 2001) and is considered the rate-limiting factor in melanin synthesis (Prota, 1992, Ito *et al.*, 2000). Dopaquinone, the initial oxidation product, is an intermediate which is used both for the production of eu- or pheomelanin. From this step forward, TYR as well as two additional enzymes TRP1 and TRP2/ DCT are involved in the synthesis of black eumelanin. The production of the yellow pigment, pheomelanin, requires another additional amino acid. The two different types of melanins are produced in two different types of melanosomes: eumelanosomes, which are rod-shaped filled with eumelanin granules and pheomelanosomes, which are spherical and the place of pheomelanin synthesis (Trinkhaus, 1948). Eu- and pheomelanosomes are said to provide slightly different conditions for the production of the two different pigments e.g. pheomelanosomes appear to lack TRP1 and TRP2/DCT (Kobayashi *et al.*, 1995).

Melanogenesis is under complex genetic control while at the same time can be affected by environmental and physiological influences. Pheomelanin production can be elevated when environmental cysteine levels are high (Smit *et al.*, 1997, Land and Riley, 2000), when conditions are more acidic (Ancans *et al.*, 2001) when TYR concentration or activity is low (Ozeki *et al.*, 1997, Ito *et al.*, 2000) or when pathways are activated that supress eumelanin production such as the agouti signalling pathway (Wolff, 2003, Takeuchi *et al.*, 2000). Higher expression of TRP1 and TRP2/DCT correlates with darker pigmentation in a number of birds such as chicken, ducks, Korean quails, pigeons and geese (Galvan and Solano, 2016). Furthermore hormones such as α-melanocyte stimulating hormone (αMSH) as well as steroid hormones like testosterone affect melanogenesis usually by enhancing the production of eumelanin (Strasser and Schwabl, 2004, Eising *et al.*, 2006).

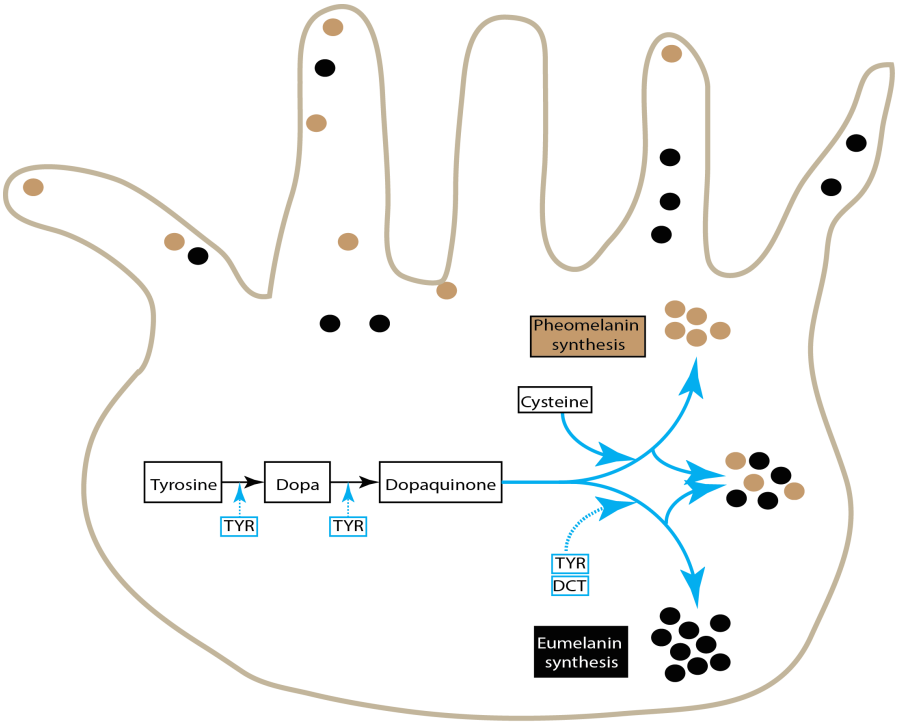

Figure 3 Mammalian skin melanocyte with schematic depiction of melanogensis. Dopaquinone serves as a substrate for both the red pheomelanin and black eumelanin. Pheomelanin production requires cysteine, eumelanin synthesis the enzymes TYR and TRP2/ DCT. The pigment packed eu- and pheomelanosomes are transported to the melanocyte dendrites and are transferred to keratinocytes using phagocytosis-like mechanism.

All cellular processes of melanogenesis including pigment deposition into keratinocytes have to be tightly matched with the maturation of the follicle during growth and moult (Yu *et al.*, 2004). The ability to generate complex colour pattern suggests mechanisms that either control proliferation and maturation of melanin producing cells and/or the selective activation and deactivation of melanin synthesis during feather growth. Melanins can therefore be used selectively to form disruptive and cryptic colouration, which require a precise arrangement of pigmented and unpigmented areas. Carotenoids on the other hand are taken up into the feather follicle along with other circulating lipids and are incorporated as a pool into single continuous patches of pigment. Carotenoid pigmentation can therefore never form patterns such as banding (Hill and McGraw, 2006).

1.2.3 The Melanocortin 1 receptor

Considering the great diversity in avian pigments and their function, understanding the molecular basis of this variation remains a challenge. Recent research has made some progress in unravelling the pathways involved in carotenoid and psittacofulvin production (Lopes *et al.*, 2016, Eriksson *et al.*, 2008, Cooke *et al.*, 2017) but up to date most knowledge has accumulated around melanin synthesis. TYR was the first enzyme to be isolated (Fling *et al.*, 1963) and loss of function mutation in this gene leads to a complete loss of melanin in skin, feather, iris and retina and is causing albinism in a broad range of taxa. Over 100 different loci can interact or contribute to pigmentation phenotypes in animals (Urabe *et al.*, 1993) and one gene, the *Melanocortin 1 receptor* (*MC1R*) has received most of the attention.

MC1R, a 7-pass transmembrane, G-protein coupled receptor, is encoded by an intronless gene usually less than 1000 base pairs (bp) long. It is expressed in mature melanocytes (Mountjoy *et al.*, 1992) and upon its activation regulates which type of melanin is produced (Figure 4). When binding its agonist, α-MSH, MC1R goes through a conformation change, which results in the detaching of a subunit of an intra-cellular coupled G-protein subunit. This detached subunit activates the adenylyl cyclase, another membrane-bound enzyme, to convert adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). The increase in cAMP levels activates cAMP-responsive element-binding proteins (CREB) in the cell to bind to their respective target genes on the DNA, activating their transcription. Most of these target genes include proteins or enzymes associated with pigmentation, such as the transcription factor MITF, which in turn increases the transcription of *TYR* (Schiaffino, 2010). A higher activity of MC1R typically results in darker pigmentation, while no or a low activity keeps the cell on a default setting to producing pheomelanin (Garcia-Borron *et al.*, 2005). In mammals a lower activity of MC1R is present when the agouti signalling protein (ASIP) is active, blocks signalling, or through mutations that render the receptor non-functional (Suzuki, 2013).

In 1996 Takeuchi and colleagues identified *MC1R* in chicken and determined that it shared 64% homology with mammals (Takeuchi *et al.*, 1996a). They later found that black chicken carry the same mutation, E92K (Takeuchi *et al.*, 1996b), which was also found to be associated with black coat colour in mice (Robbins *et al.*, 1993). These findings were the first significant evidence that *MC1R* could be involved in bird pigmentation. In mammals, αMSH is produced in the intermediate lobe of the pituitary gland, a structure missing in birds (Hill and McGraw, 2006), so prior to this discovery, it has been long assumed that *MC1R* is not fulfilling the same functional significance in birds as

it had been shown in mammals. Few studies tried to address the lack of understanding of MC1R hormonal control. It was found that ducks have α-MSH present in their anterior lobe but this was not connected to pigmentation (Iturriza *et al.*, 1980). *In-vitro* experiments with quail melanocytes suggested that αMSH has the ability to stimulate melanogenesis in those cells (Satoh and Ide, 1987) and led to the assumption that αMSH might be produced peripherally in the skin (Takeuchi *et al.*, 2003).

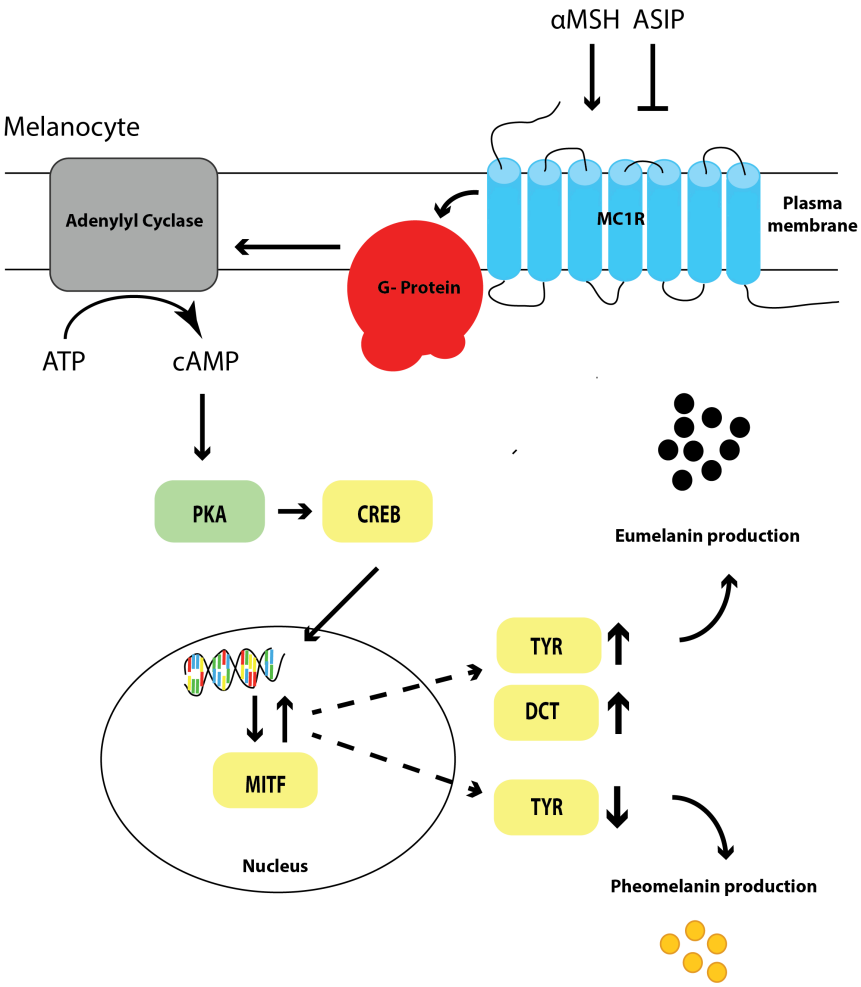

Figure 4. Signal transduction pathway mediated through MC1R. αMSH is acting as an agonist of MC1R, increasing cAMP production and leading to high transcription levels of TYR and TRP2/ DCT resulting in an increased eumelanin synthesis. ASIP is a natural antagonist of MC1R favouring the production of yellow/ red pheomelanin through decreasing *TYR* expression.

Over the years, more and more evidence regarding the involvement of *MC1R* in colour traits in birds accumulated both in domestic (Kerje *et al.*, 2003, Nadeau *et al.*, 2006, Guernsey *et al.*, 2013), as well as in wild species (Baiao and Parker, 2012, Mundy, 2005). Furthermore experimental testing showed that avian MC1R does respond to *in-vitro* αMSH stimulation and that its signalling activity often is correlated with a specific colour phenotype (Ling *et al.*, 2003, Guernsey *et al.*, 2013). In bananaquits (*Coereba flaveola*), the missense mutation E92K is associated with melanism affecting the entire body (Theron *et al.*, 2001), while other mutations in variant morphs of lesser snow geese (*Anser caerulescens*) (Mundy *et al.*, 2004) and artic skua (*Stercorarium parasiticus*) result in a gradual increase in dark pigment throughout certain body regions (Mundy, 2005). It was therefore suggested that *MC1R* induced patterning mechanisms are lineage specific and assumptions might not be easily transferable between species (Hill and McGraw, 2006, Nadeau *et al.*, 2007). Whether *MC1R* is also determining colour differences e.g. between male/female or different reproductive morphs or if it equally alters pheomelanin distribution remains poorly explored. Indeed, its potential role in within-feather pattern formation has not been investigated despite the fact that the agouti banding mediated by MC1R in the mammalian hair has been well described (Manceau *et al.*, 2011, Mallarino *et al.*, 2016).

1.3 The domestic chicken (*Gallus gallus domesticus*)

1.3.1 Domestication history of the chicken

The domestic chicken (*Gallus gallus domesticus*) is a descendent of the wild jungle fowls inhabiting the rainforests of Asia. Four *Gallus* species are known (*G. gallus*, *G. sonneratii*, *G. varius* and *G. lafayettii*) and it has been a matter of debate which of them contributed to the gene pool of today's chicken breeds. Darwin, after generating fertile offspring when crossing domestic chickens with red jungle fowls (*G. gallus*), believed in a monophyletic origin of the domestic chicken from this species (Darwin, 1868); an idea that was initially supported by genetic studies on mitochondrial DNA (Fumihito *et al.*, 1994, Fumihito *et al.*, 1996) and retrovirus insertions (Frisby *et al.*, 1979). Additional genetic evidence (Liu *et al.*, 2006, Kanginakudru *et al.*, 2008, Miao *et al.*, 2013), however, recently cast doubt on a simple monophyletic origin of this important livestock species. At present, it is believed that the chicken was domesticated on at least two to three independent occasions in different parts in Asia (e.g. in India) and that the oldest attempt seems to have occurred about

10,000 y B.P. in northern China (Xiang *et al.*, 2014). Several lines of evidence suggest that the red jungle fowl is the main ancestor of the domestic chicken with some contribution from the grey jungle fowl (*G.sonneratii*) (Xiang *et al.*, 2014, Eriksson *et al.*, 2008).

After initial domestication the tamed chicken started its triumphal procession around the world. Since chickens have none of the characteristics typical for migratory species (e.g. they don't fly well long distances, can't swim etc.), their global distribution is the result of human mediated dispersal (Storey *et al.*, 2012). They reached Europe through two main trading routes: China and Russia as well as trough Persia and Greece (Smyth Jr, 1990). Around 700 B.C. poultry keeping for food, leisure, and religious purposes was a daily part of life for the Romans. Regional chicken varieties at this time already exhibited derived phenotypes such as rose comb, muff and beards (Tixier-Boichard *et al.*, 2011). The domestic chicken was introduced to Africa at least 500-800 A.D. and is believed to have occurred on three occasions (Mwacharo *et al.*, 2013). It is likely that chickens could have reached South America from a sea route after they had reached Polynesia. The blue-egg shell mutation (*O*) might be supporting this idea: it is found in the Auracana breed in South America as well as in Chinese breeds, but not in European chicken breeds (Tixier-Boichard *et al.*, 2011).

Today, chickens of a particular breed are defined by a specific set of distinct morphological and behavioural features, which are considered to be identical-by-decent and often have a regional isolation component (Hutt, 1949). A breed can further be subdivided into different varieties, which are usually but not always defined by a specific plumage colouration or patterning (Hutt, 1949). As an example, according to the American Standard of Perfection the Plymouth Rock chicken is available in seven different varieties, all based on their plumage: barred, blue, buff, columbian, partridge, silver pencilled and white. Plumage was used early on as a parameter to distinguish breeds e.g. in the Middle Age when French chickens from the Burgundy region were characterized by the autosomal barring pattern (Tixier-Boichard *et al.*, 2011). Today, the American Poultry Associations acknowledges almost 400 different chicken breeds with several different varieties.

1.3.2 The chicken as a model for avian pigmentation

Early domesticated chickens were probably kept under protected conditions and were provided with food and shelter. Thus the occurrence of colour variants were exposed to relaxed natural selection and were most likely selected for by humans (Andersson, 2001). Chicken were mostly bred for

leisure activities such as cock fighting as well as for their eggs and meat (Smyth Jr, 1990). The regional isolation of populations and a subsequent selection for plumage traits that were perceived as appealing, eventually created the huge variation in plumage colouration and patterning observed today. This diversity is without comparison within any wild bird species – with one exception: the male ruff (*Philomachus pugnax*).

By now it is considered common knowledge that domestication traits such as pigmentation have a strong genetic basis (Rubin *et al.*, 2010) fuelling research to identify new genetic variants and pathways responsible for a certain phenotype. Despite the phenotypic diversity, each domestic chicken genetically remains a domestic chicken and differences in the genomes between two chickens are much smaller than those between two closely related bird species. This feature makes it easier to identify variants in the genome, which are directly connected to a specific appearance or behaviour. The publication of the chicken genome in 2004 (International Chicken Genome Sequencing, 2004) and its continued annotation and improvement provides an additional great resource to detect new variants. The chicken genome is small at 1.1 gigabases (Gb), compared to mammalian genomes, but still contains homologs of most genes also found in humans. Recent advances in sequencing technologies have replaced laborious Sanger sequencing, making it possible to easily sequence whole genomes of chickens, followed by bioinformatics analysis to screen for genome-wide differentiation between individuals of deviating phenotypes.

Apart from the advantage of great phenotypic diversity within a single species and the improved genome resources, domestication is providing us with another useful by-product: chickens are easy to keep and breed, so that large families of chickens segregating for certain traits can be set up quickly, increasing detection power of genetic variants. Crosses can even involve mating using the chicken ancestor, usually the red jungle fowl, as they produce fertile offspring and captive populations of the red jungle fowl are available. Furthermore fancy breeders have recorded and accumulated a wealth of classical genetic knowledge about inheritance of chicken plumage colours and patterns and carefully keep track of their own mating attempts.

In recent years, the domestic chicken has been used successfully to find new mutations and unravel genes and pathways involved in plumage pigmentation (e.g. (Gunnarsson *et al.*, 2011, Gunnarsson *et al.*, 2007, Kerje *et al.*, 2004, Hellstrom *et al.*, 2010, Kerje *et al.*, 2003, Chang *et al.*, 2006)) as well as aided in understanding morphological and cellular processes in the feather follicle (Yu *et al.*, 2004, Yue *et al.*, 2005, Lin *et al.*, 2013). While these findings cannot always be transferred directly into wild bird species, they do

enhance our general understanding about pathways and molecular processes involved in complex traits such as pigmentation.

Variant loci for plumage pigmentation can alter the intensity of the pigment or inhibit pigment production completely. The *Dominant white* locus (*I*), which completely removes black pigmentation (Hurst, 1905), or the missense mutation responsible for the dilution of dark pigmentation causing lavender colouration (Vaez *et al.*, 2008, Mayerson and Brumbaugh, 1981), are popular examples here. Chickens also exhibit a pronounced sexual dimorphism so that males and females display marked differences in plumage colouration, which can be altered by genetic variants such as henny feathering (Matsumine *et al.*, 1991). The most challenging pigment trait to study, though, is the distribution of melanin both across the body, as well as on individual feathers: a process that is likely to have a much more complex molecular mechanisms underlying than those defining the plain presence or absence of pigment.

1.3.3 Pigment pattern genes in chickens

Pigment pattern genes in chickens can both affect the distribution of pigment across the body (primary pattern) as well as on individual feathers (secondary pattern) (Kimball, 1953). Primary patterns can include several feather follicles or entire body regions, such as the belly, while secondary patterns refer to only one feather follicle. Many pigment pattern loci do not just drive pigment distribution in either of the two categories but do actually affect both.

The polyallelic *Extension* locus *E* (*MC1R*) determines the basic or zonal distribution of black eumelanin across the body of a chicken (Smyth Jr, 1990). Its effect depends on the specific allele at *MC1R* as well as the sex and the presence of other interacting loci. At least eight different alleles are recognized on the basis of inheritance of well-defined phenotypes (without the knowledge of molecular information) and are listed in their approximate order of dominance (Smyth Jr, 1990): *Extended black* (*E*E*), *Birchen* (*E*R*), *Brown* (*E*B*), *Buttercup (E*BC*), S*peckled* (*E*S*), *Wild-type* (*E*N*) and *Wheaten* (*E*WH* and *E*Y* depending on the dominance). Pigmentation intensity and distribution are decreasing from *E*E* to *E*Y* and differ remarkably between males and females. *MC1R* interacts with other colouration loci e.g. with *Dark brown* (*Db*), *Columbian* (*Co*) and *Mahagony* (*Mh*). These all restrict eumelanin to different degrees in a so-called columbian-like manner to the hackle, wing, foot and tail feather (Smyth Jr, 1990). Eumelanin restrictors are most efficient at restricting dark pigment from body feathers of females, which do not carry the *E*E* allele at *MC1R*, and from the black breast of *E*N* males (Smyth Jr, 1990). All three loci affect the visual appearance of pheomelanin.

While the role of *MC1R* in creating very dark plumage is very important, there are other factors intensifying eumelanin expression and consequently playing an important role in creating primary and secondary pattern. The best-described eumelanizing locus is *Melanotic* (*Ml*). *Ml* is an autosomal, incomplete dominant variant, which extends dark pigment in usually red areas but is not affecting down colour o chicks (Smyth Jr, 1990). Its concrete effect, however, does strongly depend on the respective allele at *MC1R*. As an example, heterozygous *Ml*/*ml*+ females with *E*WH/E*WH* show very little eumelanizing effects. *Ml* also interacts with a number of other loci affecting the formation of secondary patterns, such as single and double lacing (Table 1; (Smyth Jr, 1990)). Thus, pigment genes constitute an excellent model for analysis of gene interactions.

Secondary plumage pattern is defined by either white spotting or a specific eumelanin distribution on individual feathers (Smyth Jr, 1990) (Table 1). Most of the pattern can usually be observed in silver or gold versions depending on whether the background colour is white (silver) or red-brown pheomelanin (gold) or black. This effect has largely been attributed to the *Silver* locus (*S*), which is removing pheomelanin but not dark eumelanin from individual feathers (Smyth Jr, 1990). It was recognized early on that the inheritance of secondary pattern, such as single lacing, is rather complex, leading to the proposal that most of them are the result of interactions between different loci.

Recent research has paved the road to a better understanding of the genetic mechanisms underlying secondary pattern formation. Sex-linked barring is due to mutations in the tumour suppressor gene *CDKNA2* (Hellstrom *et al.*, 2010) and *Mo* was found to be connected to the *endothelin receptor B2* (*EDNRB2*) gene (Afonso *et al.*, 1999), and *Db* is associated with a large-scale deletion upstream of the transcription factor *SOX10* (Gunnarsson *et al.*, 2011). Very few functional validations of the proposed candidates or candidate gene mutations have been done (Hellstrom *et al.*, 2010, Lin *et al.*, 2013) but both the main function of the affected genes as well as findings from immunohistochemistry and *in-situ* experiments in feather follicles suggest that secondary pattern formation in birds involves complex mechanism of melanocyte proliferation, differentiation and migration.

Table 1. *Within-feather pigment patterns and underlying loci as predicted by Mendelian genetics. Note that Pg is predicted to be involved in the majority of pattern formation by interacting with other loci. It is therefore considered the major driver of within-feather pigment pattern formation in chicken. The X is indicative of the derived allele at the respective locus. Pg- Patterning, Ml- Melanotic, Co- Columbian, Db- Dark Brown, Mo- Mottling, B- sex-linked barring.*

Phenotype	**Colour locus**					
	Pg	*Ml*	*Co*	*Db*	*Mo*	*B*
Stippled						
Penciling	X					
Double lacing	X	X				
Single lacing	X	X	X			
Spangling	X	X		X		
Autosomal barring	X			X		
Mottling					X	
Tri colour			X		X	
Sex-linked barring						X

2 Aims of the Thesis

The overall goal of this thesis was to identify new genes and genetic variants involved in pigment pattern formation in chickens and wild birds, as well as to understand their 'mode of action'. Finding genes involved in pigmentation processes and elucidating their function will not only be ground-breaking in understanding evolution and the overwhelming extend of colour variation in birds, but can also have important implications for pigment cell-related diseases in humans.

The specific aims were:

I. To determine the genetic variant(s) underlying the within-feather pattern autosomal barring in the Fayoumi chicken breed through pooled whole genome re-sequencing and functional studies.
II. To investigate the molecular mechanisms of the four mutations in the tumor suppressor locus *CDKN2A* previously found to be associated with sex-linked barring in chickens.
III. To identify the causal variant of the three different reproductive morphs in the ruff (*Philomachus pugnax*) and better understand the underlying genetics in the light-coloured satellite morph.

3 Study Summaries

This thesis is comprised of four papers in which both genomic and molecular biology approaches were used to identify novel genes involved in pigmentation and plumage variation in birds and describe their function. In paper I, a major driver of melanin distribution on individual feathers was studied using a chicken backcross and various molecular and computational methods. In paper II, the mutations associated with the sex-linked barring pattern in chickens were investigated using molecular methods to describe pattern formation. In paper III, a 4.5 mega base (Mb) inversion was found to be associated with alternate reproductive morphs in the ruff and in paper IV the effect of *MC1R* alleles on plumage variation in the ruff was studied in more detail.

3.1 Autosomal barring in chicken is strongly associated with segregation at the *MC1R* locus (Paper I).

3.1.1 Background

In the domestic chicken, two different types of barring pattern on individual feathers have been described (Smyth Jr, 1990): autosomal and sex-linked barring. Autosomal barring presents as a semi-dominant, autosomal inherited trait, which adds a dark eumelanin bar on a brown or depigmented background (Smyth Jr, 1990). Initially it was proposed that this type of barring pattern is the result of an interaction between two loci, namely *Pg* and *Db* (Smyth Jr, 1990, Moore and Smyth, 1972, Carefoot, 1999, Carefoot, 1984) (Table 1). *Db* is considered a restrictor of eumelanin as it keeps melanin away from certain body parts such as the breast and belly. In females *Db* results in an orange tan colouration over the entire body, except for the tail. The male phenotype is

more variable and chickens shows a bright orange breast to various degrees (Gunnarsson *et al.*, 2011). An 8.3 kilo bases (kb) deletion upstream of the transcription factor *SOX10* on chromosome 1 is common to all *Db* individuals (Gunnarsson *et al.*, 2011). Although no further exploration of the mode of action of this mutation has been carried out, the authors proposed that based on similar mutations in mouse, the deletion removes a *cis*-regulatory element, which might affect *SOX10* expression in different body regions differently, leading to differences in melanogenesis in the breast and tail. Another theory suggests was that *SOX10* is enhancing the expression of *MC1R*. As a consequence of the deletion, *SOX10* is down regulated in *Db* birds and so is *MC1R*. This might cause a dosage-dependent down-regulation of *MC1R* resulting in the default production of pheomelanin on the body and breast but not in the tail. The authors did not elaborate on the potential function of SOX10 in feather follicles or the effect of the deletion on secondary patterns such as autosomal barring, but it is very likely that it does alter melanogenesis or related pathways. Hutt (1949) pointed out that autosomal barring could also be looked at as a trait, which restricts black eumelanin to a bar on an individual feather, just as dark pigment in *Db* birds is restricted to the tail (Hutt, 1949).

The second locus implicated in barring is *Pg*, for which the actual location has not been firmly established. Crossing experiments suggested that it should be found 20 cM from *Db* and 10 cM from *Ml* on chromosome 1 (Moore and Smyth, 1972). According to traditional Mendelian genetics, *Pg* is considered genetically homogenous and ubiquitously responsible for various within-feather pattern formations across different chicken breeds (Smyth Jr, 1990) (Table 1).

The Fayoumi is an old Egyptian chicken breed, which is believed to have originated from semi-rural chickens inhabiting the coastal area of the Nile and imported red jungle fowl chickens about 3,000 years ago. Fayoumis are always barred on a silver background. The barred plumage is typically visible in various degrees on the entire body, except head and neck. Fayoumis carry a rare form of the *Birchen* allele, *E*R(Fay),* at *MC1R*, which differs from the common *Birchen E*R* allele in its amino acid composition (Ling *et al.*, 2003). *E*R(Fay)* is characterized by an amino acid exchange at position 133 (L133Q), whereas the more common *Birchen E*R* allele carry, among others, E92K, the mutation previously shown to be associated with black coat colour in mice (Robbins *et al.*, 1993).

The goal of study I was to identify the genetic variant underlying the autosomal barring phenotype in the Fayoumi breed and gain some functional insights into how the autosomal barring pattern is formed.

3.1.2 Results and discussion

To verify the proposed position of the *Pg* locus on chromosome 1 and identify a candidate gene, we first generated a backcross by mating five Fayoumi females (*Pg/Pg Db/Db*) with two inbred Light Brown Leghorn males (*pg+/pg+ db+/db+*). Twelve homogeneous barred F_1 females (*Pg/pg+ Db/db+*) were crossed again with another Light Brown Leghorn male. We then examined the 365 offspring progeny both phenotypically at hatch and at 12 weeks of age, and genotyped them for the *Db* mutation. If the assumed model of inheritance of autosomal barring (with two independent dominant mutations) was correct, we should observe this phenotype in 25% of the backcross progeny, which indeed was the case. A total of 102 chickens exhibited the characteristic autosomal barring phenotype, 203 did not have any pattern and were classified as wild-type, while 60 offspring did not fit either category. Among this set of 60 animals, 28 were neither plain nor clearly barred, and 32 additional males were initially phenotyped as either wild-type or autosomal barred at hatch. At 12 weeks of age, however, they had developed a reddish taint in the body region with no visible pattern anywhere on the body. This suggested that there was another pigmentation locus segregating in our pedigree, which appeared to act epistatically to autosomal barring. Those individuals were therefore excluded from further analysis.

Additional genotyping revealed that contrary to our expectations not all chicken classified as autosomal barred carried the *Db* deletion as expected. As many as 36 of the autosomal barred chicken (about 1/3) were *Wild-type* at this locus. We also had a closer look at the group of offspring, which was neither properly barred nor plain and discovered that this group almost entirely was *db+/db+* as well (25 out of 28 offspring). The observation that *Db* apparently was not required to develop a regular autosomal barring but at the same time was also lacking in the group of chicken that showed irregular pattern, implied that *Db* is not required for autosomal barring as proposed in the literature, but is contributing (possibly among other unknown loci or genetic variants) to a more defined patterned phenotype. It appears likely that in the past breeders selected chickens with the most pronounced barring phenotype, most of them carrying the variant allele at *Db*, leading to the assumption that this locus is required for the pattern formation.

Next we used next generation sequencing (NGS) to pinpoint the location of *Pg* in the genome of our backcross progenies. We used the Fixation index (F_{ST}) and 50 kb sliding windows to look for regions with high genetic differentiation between autosomal barred and non-barred chicken. The highest differentiation between wild-type and patterned chicken was found on chromosome 11 covering a region between 18.2 – 18.9 mega bases (Mb) (the end of the

chromosome in galGal4). This region contains a number of genes involved in pigmentation or melanocyte biology with *MC1R* being the most obvious candidate. To obtain a better resolution of the region on chromosome 1 and 11, we genotyped the entire pedigree for a total of 100 SNPs located on chromosome 1, 11 and a few individual high F_{ST} SNPs on chromosome 2. We were not able to detect a completely shared haplotype for either phenotype category on chromosome 1 or 2. On chromosome 11, however, all backcross progeny exhibiting either autosomal barring or an irregular pattern, shared the same haplotype that showed no recombination from approximately 18.2 Mb until the end of the chromosome. This haplotype included the Fayoumi allele at *MC1R* (*E*R(Fay)*), which was inherited to all patterned offspring, including those which showed an irregular pattern. Not a single wild-type or plain chicken was a carrier of this haplotype. The unusual low rate of recombination in this region might suggest that a structural variant such as an inversion could be causative of this observation. Although we did not specifically test our material for an inversion, we believe that this possibility is rather unlikely since others have reported similar low recombination rates in this region (Groenen *et al.*, 2009).

A non-recombining region as observed in our pedigree makes the detection of candidate genes and mutations challenging, since the entire interval is statistically equally likely to carry the causative change(s). We used the UCSC Variant Annotation Integrator (VAI) to find mutations of interest within the non-recombined interval. We detected 42 non-synonymous mutations, which affect nine genes located within the interval of interest. However, PROVEAN, another online tool, which examined protein sequences based on their similarity, predicted that none of these mutation have a deleterious effect on the respective gene function, except for L133Q, the defining mutation for the *E*R(Fay)* allele at *MC1R*. As over 4500 non-coding SNPs were detected within the non-recombining region, it was challenging to pinpoint candidate variants, which could have functional implications. We therefore decided to evaluate the gene expression pattern in a subset of the in total 29 genes in the non-recombining region, which previously have been described to affect melanocyte biology and pigmentation including *NAD(P)H quinone dehydrogenase 1* (*NQO1*), *cadherin 1* (*CDH1*), *ww domain-containing protein 1* (*WWP*) and *MC1R*. Except for *WWP1*, all investigated loci were found to be up regulated in growing, autosomal barred feathers as compared to the wild-type. If the elevated expression was the result of *cis*-regulatory mutation, then allelic imbalance in favour of the Fayoumi allele should be detected. This however was not the case. *NQO1*, *MC1R* and *CDH1* were expressed in equal

proportions from both alleles in heterozygous, autosomal barred chicken feathers.

There are a number of chicken breeds that are classified to carry *Pg* forming all sorts of patterns, including spangling or lacing. Since our analysis left us with only one candidate mutation, L133Q, in one gene, *MC1R*, we were wondering how transferable our findings are to other *Pg* breeds. The L133Q mutation, which is defining the Fayoumi *Birchen E*R(Fay)* allele, has not been found in other chicken breeds so far. Surprisingly, we found that the Fayoumi flock we used for our experiments was not fixed for *E*R(Fay)* either. The founders of our backcross were homozygous for the mutations but the entire flock was segregating for the *MC1R* E92K missense mutation as well, without any obvious heterogeneity in the autosomal barring phenotype. We further performed whole genome pooled sequencing of chicken breeds, which were described as carrying *Pg*. This approach would have made it possible to identify an identical-by-decent (IBD) haplotype with the Fayoumi breed, in case there is one, and would make it possible to screen the entire genome for any other fixed region between those breeds. We were not able to detect any shared haplotype, either on chromosome 11 or in the entire genome. This was surprising as it contradicts the long-standing hypothesis that *Patterning* is homogenous among *Pg* breeds. The most interesting finding however was, that all investigated breeds carried an activating mutations at *MC1R* (either *E*R* or *E*B*). Functional receptor assays of different *MC1R* alleles in chicken have revealed that E92K present in the *MC1R E*E*, *E*R* and *E*B* allele lead to an constitutively active receptor (Ling *et al.*, 2003). The authors were not able to demonstrate the same effect for L133Q in *E*R(Fay*) but these experiments were conducted in mammalian cells, which might not perfectly reflect the conditions in an avian feather follicle resulting in misleading conclusions if extrapolated to birds. The experimental Fayoumi flock used for our crossing experiment has been kept and routinely monitored for over 30 years with no strong deviation or variation in the autosomal barring pattern. It is therefore likely that the two *MC1R* alleles (*E*R(Fay)* and *E*R*) have very similar effects on the autosomal barring pattern in this breed. Recent findings are supporting this hypothesis as they indicate that interactions between MC1R and its antagonist, ASIP, occur within the feather follicle (Lin *et al.*, 2013) and have been found to be strongly associated with pigmentation in chicken (Takeuchi *et al.*, 2000) and Japanese quail (*Coturnix coturnix japonica*) (Zhang *et al.*, 2013) as well as in the golden winged (*Vermivora chrysoptera*) and blue winged warbler (*Vermivora cyanoptera)* (Toews *et al.*, 2016). However, it needs to be taken into consideration that other chicken breeds carry activating *MC1R* variants, such as E92K, but do not exhibit autosomal barring or patterning. The

mutations presented in this study might therefore not be sufficient to create feather patterning on just any genetic background and further studies are necessary to understand their actions on a molecular level.

In summary, this study suggests that *MC1R* has a major effect on pigment patterning both across the avian body as well as for within-feather pattern formation. We show that autosomal barring in Fayoumi is not dependent on the variant allele at *Db* but that this locus is contributing to a more refined phenotype.

3.1.3 Future prospects

With study I, we provide a major step towards understanding within-feather pattern formation. There are still a number of questions remaining to be answered. Our genetic data strongly implicates *MC1R* in autosomal barring but in order to gain a better understanding of its role in the pattern formation process itself, it would be necessary study growing feather follicles by staining them for various melanocyte differentiation markers as well as *MC1R*, *SOX10* and *ASIP*. In which cell type is *MC1R* expressed? Is the barring pattern determined by antagonistic interactions between ASIP and αMSH? What is the cellular mechanism underlying the barring pattern? Both sex-linked barring and autosomal barring come in two varieties: a white bar on a black or brown background or a black bar on a depigmented or brown background, respectively. Are the same cellular, developmental processes underlying both barring varieties? Is the white bar presenting melanocyte progenitor cells, which in the gold varieties develop into fully differentiated, pigment-producing melanocytes? It would also be of interest to investigate autosomal barring in other chicken breeds such as the Campine breed, which is considered similar to the Fayoumi. Discovering whether the association with *MC1R* and the cellular mechanism of pattern formation are transferable to other autosomal barred chicken breeds would allow generalisation about mechanism employed in pattern formation. These studies could be expanded even further and include wild barred bird species such as some birds of prey and owls.

Our insights into pattern formation both on the body and on individual feathers will greatly benefit from gaining a better understanding about MC1R signalling in the feather follicle. The current attempts to investigate the consequences of *MC1R* mutations on the receptor functions in birds have been done in mammalian cells (Ling *et al.*, 2003, Guernsey *et al.*, 2013) and both studies are yielding controversial results regarding signalling properties and observed phenotype. The conservation of 64% sequence identity between mammalian and avian *MC1R* suggests some similarity in function (Takeuchi *et*

al., 1996a), but also leaves open the possibility to evolutionary innovations that might not have taken place in mammals or vice versa. For this purpose chicken cell lines or primary melanocytes from feather follicles could be cultivated and transfected with different *MC1R* constructs. It could also be worthwhile exploring unknown functions and pathways related to *MC1R* in feather follicles as the receptor e.g. might affect melanocyte proliferation and differentiation or even respond to different ligands or activate different downstream pathways than those affected in mammals. Furthermore, autosomal barring on different *MC1R* backgrounds have different appearances, suggesting that those differences might be the result of genetic heterogeneity at the *MC1R* locus or affect genetic modifiers such as *Db*. Autosomal barring in chicken breeds carrying the *brown* allele *E*B* exhibit more narrow bars than on the *Birchen E*R* allele background and some breeders prefer to present autosomal barred chicken being heterozygous *E*B/E*BC* (Moore and Smyth, 1972). It is therefore very important to carry out future research in order to better understand the involvement of different *MC1R* alleles in within-feather pattern formation.

Finally, we observed a subset of chickens in our backcross, for which the initially scored plumage phenotype had disappeared after 12 weeks of age, suggesting that there was another colour locus acting epistatically to autosomal barring. Setting up a pooled sequencing approach could help to identify the underlying genetic variant and functional studies - depending on their nature - could yield more knowledge about melanin pathways, epigenetic mechanisms controlling pigmentation through maturation of a chicken or the influence of hormones on plumage colour development.

3.2 Sex-linked barring is the result of both regulatory and missense mutations in the *CDKN2A* tumour suppressor gene (Paper II).

3.2.1 Background

Sex-linked barring is the second known barring pattern in chickens and defined by white bars with complete absence of pigment on a coloured background (eumelanin or pheomelanin). In contrast to autosomal barring, sex-linked barring was described as a monogenic, dominantly inherited phenotype as early as the beginning of the 20th century (Smyth Jr, 1990).

In 2010, our research group was able to identify four genetic variants: two non-coding and two missense mutations that are associated with sex-linked barring (Hellstrom *et al.*, 2010). All mutations are located in or in the vicinity of *cyclin dependent kinase inhibitor 2A* (*CDKN2A)*, a tumour suppressor gene involved in cell cycle regulation and apoptosis. In chickens the gene is located on the Z chromosome, the avian sex chromosome. In birds males are the homogametic sex carrying two Z chromosomes, whereas females are heterogametic carrying one Z and one W chromosome.

Altogether the four associated mutations form three different variant alleles: *B*B0* (referred to as *B0*), *B*B1* (referred to as *B1*) and *B*B2* (referred to as *B2*). All alleles harbour the two non-coding mutations located in the promoter and first intron respectively. The *B1* and *B2* allele carry two additional missense mutations (V9D and R10C in *B1* and *B2* respectively), both located in a functionally important part of the protein – the alternate reading frame protein (ARF). The *B1* allele is present in breeds showing the typical sex-linked barring phenotype such as the Barred Plymouth Rock and the Coucou de Rennes and exemplifies the most pronounced barring pattern of all three *B* alleles. The *B2* allele corresponds to the sex-linked dilution phenotype (B^{Sd}), which was first described by Munro (Munro, 1946). Heterozygous males and females show a light blue and barred plumage, but this is not as pronounced as that observed for chickens carrying the *B1* allele. Homozygous males on the other hand are almost completely white and reflect the incomplete dosage compensation for sex-linked genes in chickens (Smyth Jr, 1990). The phenotypic appearance of the *B0* allele, however, has been unknown so far because it has only been found in commercial egg layer like the White Leghorn, which also carries the epistatic *Dominant white* locus (Hellstrom *et al.*, 2010) and therefore exhibit a completely white plumage.

The 2010 study did not determine if indeed all four mutations are involved in creating the striping pattern. Even if a phenotype of the *B0* allele was unknown at this time point, the fact that the two non-coding mutations were not detected in any *Wild-type* haplotype suggested that either one or both could be functionally important. In 2010, Hellström *et al*. performed an electrophoretic mobility shift and a luciferase reporter assay in an attempt to elucidate a potential regulatory effect of the two non-coding mutations in the promoter and first intron of *CDKN2A* but they were unable to detect any differences between the two non-coding SNPs or the different alleles. Based on the involvement of ARF in cell cycle regulation, the authors proposed that the mutations could lead to premature apoptosis of melanocytes during active feather grow, leaving behind a white bar. The following black bar could be the result of a replenished melanocyte pool producing pigment until it is exhausted

at which point the cycle starts all over again. This idea was based on the observations that during white band formation hardly any melanocytes are present in the growing feather shaft and that cultured pigment cells from sex-linked barred chickens die five times earlier if compared to the wild-type (Bowers, 1988). In 2013, Lin and co-authors published a study in which they described the mechanisms of a number of feather pattern from different chicken breeds. They divided the white and black bars of sex-linked barred chickens into four different phases: the early black, late black, early white and late white phase. Each phase represents a gradual loss of melanocyte progenitor cells up to complete absence in the white bar. Based on a negative terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining they proposed the possibility that the lack of melanocytes was not due to apoptosis, but instead hypothesized that the mutations cause an aberrant feedback mechanism leading to premature differentiation of melanocytes.

The link between ARF, melanocytes and apoptosis made in these studies so far is not arbitrary. ARF has a definite role in melanocyte biology, which is exemplified by the fact that mutations affecting this protein are the most common cause of familial forms of melanoma in humans (Dracopoli and Fountain, 1996, Hussussian *et al.*, 1994, Kannengiesser *et al.*, 2007). ARF associates with a number of proteins promoting their posttranslational modification like sumoylation and phosphorylation to activate or deactivate their function (Gallagher *et al.*, 2006, Herkert *et al.*, 2010). ARF interacts with mouse double minute 2 protein (MDM2) and protects the transcription factor p53 from degradation, which is one of the most studied pathways in mammals (Haupt *et al.*, 1997, Sharpless, 2005). Among the numerous p53 downstream targets, the cyclin-dependent kinase inhibitor ($p21^{CIP}$) and Bcl2 associated X protein (BAX) are considered the key enzymes in cell cycle arrest and apoptosis, respectively. Similarly, the chicken ARF does interact with MDM2 and is able to protect the transcription factor p53 from degradation (Kim *et al.*, 2003).

The aim of this study was to investigate whether both non-coding and missense mutations are indeed involved in the barring phenotype and to establish a model of the pattern formation.

3.2.2 Results and discussion

The fact that the two non-coding mutations associated with sex-linked barring are present in all three *B* alleles, but not on any *Wild-type* haplotype, suggests that either one or both could be functionally important. To explore this hypothesis, we first generated a chicken cross between a White Leghorn line,

which was known to carry the *B0* allele, and red jungle fowl females. The set-up removed the *Dominant white* allele from the genetic background and revealed that chickens hetero- or hemizygous for *B0* indeed show a barring pattern that is notably lighter than the one observed for either of the two other alleles (*B1* and *B2*). Therefore, we chose to name the allele '*sex-linked extreme dilution*'.

The notion that *B0* alone appears sufficient to cause a faint, yet clear barring pattern, was further verified on the molecular level. Gene expression data from growing feather follicles revealed that *CDKN2A* expression is 2 to 3 fold elevated in sex-linked barred feathers from birds carrying the *B0* or the *B2* allele. The high *CDKN2A* expression was not the consequence of the phenotypic differences between mutant and wild-type feathers, but rather the result of allelic imbalance in favour of the mutant allele, providing additional proof for a *cis*-regulatory effect of the non-coding mutations.

As both chickens carrying the *B1* or the *B2* allele show a deviation from the 'sex-linked extreme dilution' phenotype, we assumed that the missense mutations in the gene could further modulate the appearance of the pattern. We used two biophysical methods in addition to a luciferase assay to explore a potential effect on protein-protein interactions of the gene product ARF. All three methods were in agreement with our hypothesis that the two missense mutations are interfering with the ARF-MDM2 interaction. Our data furthermore showed that V9D (*B1*), causing sex-linked barring, had the most severe, disruptive effect.

The functional data verified that most likely all SNPs previously found to be associated with sex-linked barring, are likely to be involved in creating the feather pattern. We were not able to decipher whether both of the non-coding SNPs are regulatory or if it is just one and the other one hitchhiked due to the close genetic proximity. Further studies would be required on this part. We propose that either one or both SNPs lead to a tissue-specific, *cis*-acting up-regulation of *CDKN2A* expression, which results in more ARF to protect the transcription factor p53 from degradation. p53 in turn most likely activates downstream targets involved in cell cycle regulations and apoptosis. In line with previous reports (Lin *et al.*, 2013), we were not able to detect any sign of apoptosis in sex-linked barred feathers. Instead immunohistochemistry and *in-situ* experiments suggested that melanocyte progenitor cells prematurely leave the cell cycle and differentiate into pigment-producing cells. This likely leads to a lack of pigment-producing melanocytes and the formation of the white bar. New melanocyte progenitor cells are recruited eventually to produce pigment again, which is resulting in the pigmented bar. The process continues in a cyclic pattern, creating alternating pigmented and unpigmented bars.

It is remarkable how well the molecular characterization corresponds to the observed phenotypic variation at the *B* locus. The regulatory mutations as present in the *B0* allele reduce the number of melanocytes and therefore the pigmentation intensity most effectively, resulting in the lightest striping pattern – 'sex-linked extreme dilution' – observed for the *B* locus. We have not been able to evaluate the barring pattern of chickens homozygous for *B0* but we assume that they have very little pigmentation. The two independent missense mutations in ARF counteract the over-expression of *CDKN2A*. Despite the higher number of *CDKN2A* transcripts, the impaired interaction of ARF with MDM2 leads to less p53 and less melanocyte progenitor cells to leave the cell cycle. One of the missense mutations, V9D, is most disruptive, allowing more melanocytes to continue proliferating and eventually produce pigment. This leads to very regular, distinct and darker pigmented bands and the typical sex-linked barring phenotype present in modern chicken breeds.

Furthermore our findings suggest that sex-linked barring has evolved successively with the non-coding mutations occurring first, followed by two independent events creating the missense mutations on the same haplotype as the non-coding variants. It seems that this 'evolution of alleles' is a common measure to enhance or refine phenotypes of various kinds and has both been observed in domestic animals (Rubin *et al.*, 2012, Kerje *et al.*, 2004, Imsland *et al.*, 2012, Karlsson *et al.*, 2007) as well as in natural populations such as Darwin finches (Lamichhaney *et al.*, 2015).

In study II, we showed that both *cis*-regulatory and missense mutations previously associated with sex-linked barring in chickens indeed do contribute to this phenotype. We illustrate how such combinations can create specific phenotypic effects and further were able to describe a mechanism of barring formation on individual feathers.

3.2.3 Future prospects

Although our study has been one of the first investigations describing the molecular basis of how a specific pattern on individual feathers can arise, some parts of the pathway remain rather fragmentary, such as how ARF is regulating the proliferation and differentiation of melanocytes. If it is indeed the p53 pathway determining the developmental fate of melanocyte progenitor cells, which target genes initiate the cell to leave the cell cycle? Does the mechanism involve G1-S or G2 arrest? We were unable to see expression changes for $p21^{CIP}$ but detected differential expression of *pleckstrin homology like domain family member 3 (PHLDA3)*, another downstream target of p53, as well as cell cycle regulator *tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase*

activation protein beta (YWHAB). The biological significance of these findings, however, remains to be understood and validated. A larger sample set for gene expression evaluation could validate the involvement of those two gene candidates. Transfection experiments involving overexpression of certain target genes in melanocytes followed by fluorescence activated cell sorting (FACS) could help to evaluate the effect of specific candidate genes on cell cycle progression in melanocytes. It is also possible that the mutations in *CDKN2A* affect the interaction between ARF and other proteins involved in melanocyte biology, which we are not yet aware of and which may be specific to birds. As an example in mammals, p53 induction can also be mediated by the ARF-binding protein 1/ Mcl-1 ubiquitin ligase E3 (ARF-BP1). ARF-BP1 directly binds to and ubiquitinates p53 and its activity is inhibited by higher expression of ARF (Gallagher *et al.*, 2006). Silencing of ARF-BP1 results in transcriptional activation of various p53 targets and could potentially lead to similar observations.

Furthermore, our findings were consistent with the creation of the white bar but the reoccurrence of the pigmented bar could not be sufficiently addressed. How are the melanocyte progenitor cells recruited to the barbs again? How do the melanocyte progenitor cells cycle in a feather? Are there 'migration waves' or continuous recruitments from the feather base? Or is there a signal triggering the migration up the feather shaft? Our immunohistochemistry and *in-situ* data provide limited implications because the feather follicles were poorly stage matched and sections were done randomly through the entire feather shaft. The distribution of melanocytes and their proliferative states however are spatially restricted and depend on the phase ('black' vs. 'white') of the respective feather part. Extended immunohistochemistry and *in-situ* experiments better taking these niche occupations into account as well as involving the visualisation of other proliferation markers in growing feather follicles, could give more answers. Another option would be to track the proliferation behaviour of melanocytes at different stages of the feather development employing BrdU labelling. In the case of *CDKN2A* interactions, double staining for a melanocyte marker and *CDKN2A* could be applied to confirm that *CDKN2A* is acting in melanocytes directly and not in the surrounding keratinocytes, which also have been shown to be involved in the formation of pattern.

It could also be of interest to investigate, which factors bind to the regulatory elements in the promoter and/or first intron of *CDKN2A* and how they are affected by the mutations. Do the mutations create a novel motif or does it interfere with the detachment of the factors so that the expression from this locus is affected? Preliminary experiments with electrophorectic mobility

shift assays using mouse melanA cells were not able to detect any differences between the different SNPs and alleles (Hellstrom *et al.*, 2010). It is very well possible that this is due to missing factors in the mammalian cell line they have used and that utilizing a different cell model e.g. primary cultivated melanocytes from sex-linked barred chickens, could give a different result.

On a broader perspective it is rather remarkable that the chickens carrying mutations affecting an important tumour suppressor locus do not appear to have a higher incidence rate of cancer or other health related problems. Even though the up-regulation of *CDKN2A* is tissue specific, the chickens do carry a missense mutation, which interfere with the function of the gene product. It is possible that in chickens the overall function of ARF is still sufficient in other tissues. Alternatively, the missense mutation could affect a pathway specific to feather follicles, or that different functions of ARF are mediated by different parts of the protein. Another scenario could be that slightly deleterious effects associated with sex-linked barring are compensated for by other mutations. All three hypotheses require experimental proof.

Even though barring is a very common pattern observed in wild birds, it is rather unlikely that these are all due to mutations in the *CDKN2A* locus. It can still be worthwhile to investigate the occurrence of mutations in and around this locus in other barred birds. As an example zebra finch males display a distinct barring pattern and carry a variant amino acid both at residue 9 and 10 of the ARF protein (Hellstrom *et al.*, 2010). With the recent advances in NGS technology and the sequencing of entire genomes, the readily available 48+ bird genomes (Jarvis *et al.*, 2014) should be of sufficient quality to easily examine variation at and around the *CDKN2A* locus in particular in owls and birds of prey species where barring patterns are very common. Immunohistochemistry or gene expression evaluation in growing feather follicles of these species may indicate whether *CDKN2A* is playing a role in creating those patterns too.

3.3 An inversion is associated with variant male reproductive strategies in the ruff (*Philomachus pugnax*) and lightly coloured ornamental feathers in the satellite morph (Paper III and IV).

3.3.1 Background

The ruff (*Philomachus pugnax*) is a medium sized wader with a complex mating system and great plumage variation. Males belong to either of three

reproductive strategies or 'morphs': 'independents', 'satellites' or 'faeders'. The vast majority of the males are independents (80-95%), which occupy and aggressively defend small territories on leks (Hogan-Warburg, 1966, van Rhijn, 1991, Höglund and Alatalo, 1995, Widemo, 1998). About 5-20% of the males are satellites, which are slightly smaller in size and join the independents on the courts to attract females (Widemo, 1998). They are non-territorial and submissive and compete for matings with the independents. Faeders are rare (<1%), female mimicking males, which were long debated to exist (Hogan-Warburg, 1966) but that were only recently described in more detail (Jukema and Piersma, 2006). They visit the leks to sneak matings with the females attracted by the independents and satellite males.

Most of the year, ruffs are exhibiting rather unspectacular plumage pigmentation, hardly indicating any differences between male and female or the different male morphs. During breeding season however, independents and satellites grow elaborate ornamental feathers around the neck (ruff) and on the head (head tufts) whereas faeders maintain their non-ornamental, female-like plumage all year around. The colouration of the plumage is extensive and has been subject to many studies (Höglund and Lundberg, 1989, Ekblom *et al.*, 2012, van Rhijn J, 2014). The ornamental feathers can be plain-coloured varying from white or ivory over rust, brown and black. They can also exhibit irregular or regular patterns such as dots and spots or bars of different width. In both morphs, ornamental feathers around the neck can be uniformly-coloured or differently-coloured and distributed in a mosaic like fashion. The head tufts and ornamental feathers around the neck vary independently in colouration and patterning within one individual in both independents and satellites (Dale *et al.*, 2001, van Rhijn J, 2014). Despite this great variation there is a morph-specific component to the colour characteristics. Independents show the greatest pigmentation variation among the morphs, and are predominately dark and more likely to develop regular pattern such as barring. Satellites, however, are lighter in colour (Hogan-Warburg, 1966, van Rhijn, 1991), such that completely black feathers are rare, regular patterns such as barring rather uncommon. Ornamental feathers in satellites are typically white or rust with some irregular dots.

It has been shown that both the *Satellite* as well as the *Faeder* allele are inherited in an autosomal, dominant fashion (Lank *et al.*, 1995, Lank *et al.*, 2013). It has also been reported that the *Faeder* locus responsible for the lack of ornamental feathers in the rare female mimic morph, is associated with a microsatellite marker, which was predicted to be located in close vicinity of the *MC1R* gene (Farrell *et al.*, 2013). Dale *et al.,* 2001, proposed that the general variability in plumage colouration and patterning of ornamental feathers could

be used for individual identity signalling. They argued that the display of ruffs is silent because the plumage could replace the vocal signals used in other species. In such a scenario, mutations promoting individual recognition by pigmentation alteration would be positively selected for as long as they are not affecting other major survival and reproductive traits (Dale *et al.*, 2001). There are over 100 genes implicated in pigmentation in vertebrates (Barsh, 1996, Hoekstra, 2006) but only few have been studied in the ruff. When this thesis work started, the precise genetic and molecular basis of the morph specific pigmentation variation in the ruff was still a mystery.

The aim of study III was therefore to first identify the genetic basis for the three reproductive strategies in the ruff and then further explore candidate genes in study IV, to shed light on the genetic basis of morph specific pigmentation variation between independent and satellites.

3.3.2 Results and discussion

For study III, we first generated a high quality reference genome from one single independent male kept at Helsinki zoo, Finland, using Illumina HiSeq 2000 sequencing technology. The genome was estimated to be 1.23 Gb in size, which is considered well in line to what has been reported for other birds (Tuttle *et al.*, 2016). In a next step we performed whole genome re-sequencing of 15 independent and 9 satellite males exhibiting the typical dark and light pigmentation differences in their respective ornamental neck feathers. A genome-wide screen for differentiation between the two sample sets using F_{ST}, revealed a highly differentiated, 4.5 Mb region on scaffold 28. Using BreakDancer, we were able to further describe the genetic architecture of this region as a 4.5 Mb inversion on the satellite chromosome. A diagnostic test verified that all 24 satellites contained in our total sample set, were heterozygous for the inversion and that all 112 independents were homozygous for the non-inverted allele. Since the inversion is disrupting conserved synteny in birds, we could assign the ancestral state to the *Independent* allele and answer the long standing question on which morph came first (Jukema and Piersma, 2006).

We performed a similar analysis after generating a 30x coverage-genome of a faeder individual. Comparing *Faeder* and *Independent* allele revealed an equal amount of differentiation across the entire 4.5 Mb inverted region whereas a comparison of the *Faeder* and *Satellite* chromosomes showed a variable degree of differentiation within the inversion region, which was also apparent when *Independent* and *Satellite* chromosome were compared with each other: two regions of lower F_{ST} (but still higher in value than the

background F_{ST} of the remaining genome) were interrupted by a short region with high F_{ST}. Interestingly, this pattern was the exact mirror image of what we observed in the *Faeder-Satellite* comparison. The pattern is consistent with a scenario in which the *Faeder* allele arose first by an inversion covering the entire 4.5 Mb region about 3.87 ± 0.15 million years ago. About 520.000 ± 20.000 years ago and through two additional inversion events within the inverted region, the *Satellite* allele arose by a recombination event between *Independent* and *Faeder*-like allele.

We further examined the genetic architecture of the inversion to find candidate mutations and genes, which are likely to be involved in the phenotypic differences between the morphs. This is a challenging task due to the complete linkage disequilibrium within the inversion. Furthermore the region is covering 90 genes and harbours a large number of sequence differences between the alleles. Three important features, which are most likely contributing to the phenotype, are listed below:

I. One of the breakpoints of the inversion disrupts the gene encoding for centromere protein N (CENPN), a protein that has been shown to be responsible for centromere assembly during mitosis (Foltz *et al.*, 2006). Pedigree data of a captive ruff population proved that the inversion is in deed a recessive lethal as predicted (Kupper *et al.*, 2016). In order to compensate for the lethality in homozygotes the *Satellite* allele must provide a 5% fitness increase to be maintained at an allele frequency of about 5%. The fact that the inversion can only be present in the heterozygous state also suggests that only mutations with some degree of dominance can contribute to the phenotype.

II. We further discovered three deletions (3.3, 5.2 and 17.6 kb) present in both the *Satellite* and *Faeder* alleles but not in the *Independent* allele. Two deletions remove evolutionary conserved elements around two genes involved in hormone metabolism: *hydroxysteroid (17-β) dehydrogenase 2* (*HSD17B2*) and *short-chain dehydrogenase/reductase family 42E*, *member 1* (*SDR42E1*). *HSD17B2* is catalysing the conversion of 17β-hydroxysteroids (e.g. testosterone) to 17-ketosteroids (e.g. androstenedion). Since both androgens and oestrogens have their highest affinity to their receptors in the 17β-hydroxylsteroids form, the enzyme is considered to regulate the biological activity of sex-hormones (Henderson *et al.*, 2003). We propose that these deletions remove *cis*-regulatory elements, which alter the expression pattern of one

or both genes. In 2016, Kupper *et al*. could show that the testosterone concentrations in blood plasma samples of independent males were elevated around breeding season, whereas satellites and faeders show no such peak but instead an increase of androstenedion level. The differences in hormone levels most likely contribute to phenotypical and behavioural differences between male morphs. Testosterone is known to increase muscle and body size and is also implicated in aggressive behaviour, both traits drastically differing between independents and the two variant male morphs. The exact mechanisms however require further experimental studies.

III. A large number of missense mutations are present in the *Satellite* and *Faeder* allele. Four of them were of particular interest to us as they are located within the coding region of the satellite *MC1R*. *MC1R* is the most interesting candidate gene within the inversion in regard to the pigmentation differences between the morphs. All four mutations (V105L, R149H, H207R and R303W) represent the derived state as they are highly conserved between mammals and birds. H207R is the only *MC1R* polymorphism shared between *Satellite* and *Faeder* alleles and the latter one contains three additional sequence variants (R11C, R307Q and V309M). Sanger sequencing revealed complete association between the morphs and the respective mutations detected in our initial whole genome re-sequencing approach. The functional consequences of the mutations warranted further functional studies.

We propose that the inversions that led to the evolution of the *Faeder* and *Satellite* alleles, most likely had some phenotypical effects themselves. The inversion may alter chromatin interaction of genes within the inversion or even outside the inversion but close to the breakpoints. This is a possible scenario for cytochrome b5 (CYB5B), a membrane bound hemoprotein functioning as an electron carrier for several membrane bound oxygenases. The gene is located in the near vicinity of one side of the breakpoints and is implicated in biosynthesis of glucocorticoids and sex steroids (Soucy and Luu-The, 2002). We further suggest that the two deletions located in the vicinity of *HSD17B2* present both in the *Faeder* and *Satellite* alleles, are affecting hormone metabolism and hormone-related traits such as behaviour. Additional mutations occurring within the inversion over a period of up to 3.8 million years were further driving the differentiation between the morphs as the one proposed for *MC1R* in the *Satellite* allele.

Large inversions are often implicated as a genetic basis for complex traits providing ecological adaptations (e.g. (Prevosti *et al.*, 1988, Wang *et al.*, 2013)). As a cluster of physically linked genes, they are often termed 'supergenes' and are inherited like a unit rather than individual genes (Schwander *et al.*, 2014). To maintain a supergene, recombination events need to be rare. While the physical closeness of the genes itself is of advantage, there are several mechanisms, which can reduce recombination effectively. Inversions are considered one of the most fundamental ones. A very intriguing example of a supergene is the >100 Mb inversion described in the white-throated sparrow (*Zonotrichia albicollis*; (Thorneycroft, 1975, Huynh *et al.*, 2011)). In this species reproductive morphs are also associated with both plumage and behavioural variation.

In study III, we have shown that the two variant reproductive morphs, satellite and faeder, in the ruff are associated with a large inversion and presented candidate genes and mutations, which are likely to play a role in the complex phenotype observed in this species.

In study IV, we further expanded our previous work by focussing on the genetic basis of pigment intensity differences in the independent and satellite morphs. We were wondering whether the *Independent MC1R* allele (herein referred to as *iMC1R*) and *Satellite* allele (herein referred to as *sMC1R*) could play a major role in explaining these differences. In contrast to stable colour polymorphisms such as the melanism observed in the red-footed booby, a bird species in which one colour morph is maintaining the same pigmentation their entire life (Baiao and Parker, 2012), ruffs are essentially undistinguishable from each other outside the breeding season. This suggests that all morphs have the same or almost the same ability to produce eumelanin but that pigment production might be differentially regulated during breeding season and/or in different feather types or body parts. We were reasoning that in order to be causal, the *sMC1R* allele must have a dominant negative effect, which could be the result of a combination of regulatory and coding changes. To ensure proper pigment production outside the breeding season, this dominant negative variant could be exclusively or higher expressed either during breeding season or in certain feather types of satellite males only.

To test this hypothesis, we collected differently coloured, growing feather follicles from the ruff of both independent and satellite males. Outside the breeding season, we collected grey coloured primary coverts (wing) from the same males, as well as under tail coverts (tail), which are white in all individuals. Quantitative polymerase chain reaction (qPCR) revealed that *MC1R* was expressed at a higher level in white, rust and black ornamental

feathers of satellites as compared to ornamental feathers from independent males; a trend that was also true for both sets of non-ornamental feathers. The most pronounced difference between independents and satellites was observed in black ornamental feathers. Higher expression of *MC1R* is usually associated with a higher production of dark pigment (Garcia-Borron *et al.*, 2005) and melanism. Our expression data suggested that in order to produce the same amount of pigment, *MC1R* had to be more highly expressed in feathers of the same colour in satellites than in independents. We were wondering if the elevated expression levels could be the result of *cis*-regulatory mutations located on the *Satellite* allele and performed allelic imbalance assays on feathers from satellite males. The *iMC1R* allele was present in a higher percentage in coloured feathers as compared to the derived *Satellite* allele. This might imply that sMC1R homomers or dimers are functionally impaired and in order to produce pigment need to be replaced by the homodimer wild-type version of the *Independent*.

We used PROVEAN, an online tool generating an alignment based score, which measures the sequence similarity of a sequence of interest to a protein homolog before and after introduction of a genetic variant (e.g. a SNP) and generates a score, which predicts an effect of the respective mutation on protein function. All four mutations in s*MC1R* were predicted to affect MC1R function. We proceeded by measuring cAMP production in HEK 293 cells, which were transiently transfected with either the *sMC1R* or *iMC1R*. Cells transfected with only the *iMC1R* exhibited a normal, dosage-dependent response to αMSH stimulation, which in total was a bit lower than what was observed for human MC1R. Cells transfected with only *sMC1R* did also respond to αMSH exposure but had much higher cAMP basal levels and a much stronger cAMP production response after binding its ligand. These findings suggest that in a homozygous state, sMC1R is constitutively active. Even though a co-transfection of both *iMC1R* and *sMC1R* appears to abolish the constitutive effect of this allele, this finding was unexpected. Most of the mutations described for both mammals and birds around the amino acid sites where we observe the variant SNPs in the *sMC1R* allele, would predict a fair or lighter phenotype, which is expected to be associated with alleles with reduced signalling. As an example in humans the variant R151C and I155T are not just associated with a red hair phenotype but in transient transfection experiments, those mutations are connected with a decreased cAMP basal level and low response to ligand stimulation (Beaumont *et al.*, 2007, Schioth *et al.*, 1999). Two other mutations closely located to the R303W polymorphism in the ruff MC1R, are associated with hypopigmentation in dogs (Newton *et al.*, 2000) and Antarctic fur seal (*Arctocephalus gazelle*) (Peters *et al.*, 2016). In 2012,

Baiao and Parker found H207R to be associated with melanism in the red-footed booby, a large seabird species. However, they also reported another amino acid variant, V85M, which is located in the first transmembrane domain of the receptor. This variant is also associated with melanism in the lesser snow goose (Mundy *et al.*, 2004) and close to E92K, which has been shown to cause the receptor to be constitutively active (Ling *et al.*, 2003). The authors pointed out that they could not distinguish between the functional effects of either of the two mutations as they are closely linked to each other but they speculated that V85M is the main contributor to the phenotype.

We further decided to explore the possibility that sMC1R might respond differently to a prolonged presence of its agonist, such that cAMP production would decrease more rapidly as compared to cAMP production emanating from iMC1R signalling, a situation that might mimic physiological conditions in ruffs during breeding season. The process is called desensitization and is a mechanism that leads to an attenuation of receptor responsiveness. This decrease can be mediated through a number of molecular processes (Ferguson, 2001) and often occurs in constitutively active receptors. Cells transfected with *iMC1R* responded with a reduced production of cAMP after 60 min of stimulation with αMSH. A similar pattern was observed for co-transfections using *sMC1R* and *iMC1R* as long as the plasmid transfection ratio was exceeding 1:2. Cells transfected only with *sMC1R* did not show any attenuation of the receptor function. The results suggest that the wild-type version of MC1R is abolishing the alternated function of sMC1R in our cell type model.

Finally, we examined the surface expression of iMC1R and sMC1R under different co-transfection ratios and discovered that sMC1R is transported to the cell surface more efficiently than the wild-type. This finding is unexpected since most fair or light phenotypes in humans are associated with a lower surface expression of MC1R (Beaumont *et al.*, 2005, Sanchez-Mas *et al.*, 2005). The reduced presence of mutant MC1R on the cell surface are ultimately considered the reason for reduced cAMP levels and less pigment production. There are, however, also variants in human MC1R (D294H), which are associated with red hair and which show a higher surface expression than the wild-type receptor (Beaumont *et al.*, 2005). These authors suggested that since this variant has been linked to an inefficient G-protein coupling, it also has reduced basal internalization, which might result in higher surface expression.

Receptor studies on mammalian MC1R have been routinely performed in HEK 293T cell models and yielded valuable information on the functional consequences of variant alleles. With a sequence identity of 64% between

human and chicken *MC1R*, it is possible that avian MC1R functionality is compromised in mammalian cells e.g. through altered protein folding and trafficking. Our results suggest that our cell model can be utulized to generate pharmacological information on avian receptor function, such as cAMP production, but is most likely not useful to explore more complex regulatory mechanisms like desensitization or cell surface expression. Since all reproductive morphs in the ruff are able to produce dark and light pigment outside the breeding season, it is reasonable to assume that more fine-tune mechanisms are employed to create the great plumage variation observed. Further studies involving avian cells, possibly even primary cultured melanocytes, will be necessary to answer this question.

In study IV, we describe allele-specific effects on both gene expression and functionality of the pigment gene *MC1R*, which are most likely contributing to the dark and light plumage variation observed in the satellite and independent morphs. Our results indicate that mammalian cells may not be a suitable model to fully understand differences in MC1R performance between these alleles.

3.3.3 Future prospects

In study III, we were able to demonstrate that an inversion is associated with the two alternative reproductive morphs in the ruff. However, how the phenotypic differences are encoded by the inversion itself is not known, except that the disruption of the *CENPN* gene makes this chromosomal re-arrangement a recessive lethal. Does the large-scale inversion itself already cause phenotypic effects? Which genes within the inversion or in the flanking regions are involved in creating the great visual and behavioural variation in this species? Which sequence polymorphisms are associated with the alterations and what is their underlying molecular basis? Answering these questions might contribute a lot to our understanding of how supergenes in general can generate complex phenotypes.

We have collected a number of tissues of both satellite and independent males during breeding season and extracted their RNA for RNA-seq. Evaluating the total as well as allele-specific expression of candidate genes within the inversion such as *CYB5B* and *HSD17B2*, will aid in identifying genes affected by the inversion or other regulatory mutations present on the *Satellite* allele. The results can be verified by testing a chosen number of hypothetical regulatory elements in a transfection experiment involving appropriate cell lines and e.g. a luciferase reporter assay. This latter approach could be particular useful to explore the consequences of the three deletions on the *Satellite* and *Faeder* allele in the vicinity of *HSD17B2*. We also found a

large set of missense mutations on the *Satellite* allele e.g. in *SDR42E1*, which could be explored by using appropriate functional assays.

An in depth investigation on the S*atellite* allele will lead to a better description of this allele. In study III, we were able to identify some genomic features unique to the satellite, but the precise breakpoints of the allele caused by the second recombination event, remain to be validated experimentally. Furthermore, it remains speculative which genes could be involved in determining this specific phenotype. It is interesting that the modern *Satellite* allele is 'restoring' some of the independent features not present in modern faeders, such as the development of ornamental feathers and a more coloured plumage during breeding season (albeit not as darkly coloured as seen in modern independents). A comparative investigation of both alleles might lead to the discovery of so far unknown genes and pathways involved in these traits and the proposed functional studies outlined above could be used to validate gene function and candidate mutations.

Apart from addressing the functional effects of the three different alleles, their broad scale evolution and fitness consequences could be further highlighted by answering questions such as: How do the inversions evolve over time? Do satellite females avoid mating with faeders and satellite males? Since the inversion is a recessive lethal, recessive, negative mutations will accumulate in both the *Faeder* and *Satellite* alleles due to a lack of purifying selection. This might lead to functional degradation of genes in a similar matter as described for the evolution of sex chromosomes (Graves, 2001). Functional degradation of the derived alleles in the ruff could be investigated by using estimates to calculate the presence of non-synonymous mutations within the inverted regions as compared to the remaining genome. An excess within the inversion might be evidence for functional degradation. Functionally, this can be reflected by an overall reduction of gene expression within the inverted interval in the *Satellite* or *Faeder* allele without an overall up-regulation of the respective *Wild-type* allele. We have already generated high quality DNA sequences of a single satellite male, which will give enough resolution to address this question. The samples collected for RNA-seq will give information on the expression of genes within the inversion.

By exploring the 'mode of action' of mutations in *MC1R* of satellite male morphs in the ruff, we have made an important step in evaluating *MC1R* variants and their actual functional contribution to colour pattern variation in natural avian populations. The results obtained however were not fully conclusive and raise manifold questions.

We observed expression differences between *iMC1R* and *sMC1R* in similar coloured feathers of satellite males but it remains unknown whether the reduced expression of *sMC1R* is caused by a mutation in a regulatory region of the gene or if the coding mutations themselves affect gene expression. The first question can be addressed by identifying non-coding regions around *MC1R*, which are conserved among the 48+ sequenced birds and search for genetic variants within the *Satellite* allele in those positions. The respective regions could be transfected into cell lines or primary melanocytes and evaluated for their effect on gene expression e.g. by using a luciferase reporter assay. Furthermore, it is feasible that hormone concentrations locally present in the skin might affect both *iMC1R* expression as well as pigmentation differently. It has been shown that whereas independents have an increase in testosterone during mating season, satellites produce more androstendion. The androstendion level in the faeder is even higher whereby pigment intensity is the lowest. Granted the faeder is genetically different from the satellite, but those observations could suggest an effect of androstendion and testosterone on colouration and *MC1R* expression. Another hypothesis could be that testosterone is more efficient in activating gene expression related to pigmentation than androstendion. Both hypotheses are testable *in-vitro* by growing primary melanocytes from feather explants of independent and satellites and exposing them to testosterone and androstendion. The cells could later be harvested and analysed for *MC1R* expression or evaluated for melanin content.

Our study did also not take into account that there might be alternative *MC1R* transcripts in birds, which could be of functional significance for colour polymorphisms. In mice three different *MC1R* transcripts are known to be expressed depending on whether the melanocyte is untreated, stimulated by αMSH or by its antagonist ASIP (Rouzaud *et al.*, 2003). If the cell is exposed to its agonist, it will produce two *MC1R* transcripts in different ratios, which increase the number of receptors on the cell surface. If its antagonist binds, a third transcript is generated, which has a drastically decreased translation efficiency and leads to a reduced number of receptors. While these variants change the structure of the murine 5'UTR, skin areas of different colouration in cattle show an altered 3'UTR of *MC1R* (Rouzaud *et al.*, 2003). To our knowledge no study so far has investigated alternative transcript variants of *MC1R* and their potential functional effects in birds. The experimental procedure would include performing 3' and 5' RACE amplifications of mRNA or RNA-seq from different feathers.

It is puzzling that the expression level of both *iMC1R* and *sMC1R* in rust feathers is higher than in the black feathers of the respective morph. The

production of pheomelanin is associated with a low activity of MC1R usually mediated through ASIP. In other species exhibiting pheomelanin colouration, *MC1R* was rather low or not at all expressed (e.g. (Zhang *et al.*, 2013)). Since mRNA levels do not necessarily have to correspond to protein levels, it would be useful to validate the observed ratios for the actual receptor by using e.g. western blot techniques and specific antibodies. A possible hypothesis could be that an alternative transcript variant similar to the mouse, codes for a receptor that is less efficiently translated. An additional way to explore the unexpected high expression of *MC1R* in rust feathers and its potential functional implications would be to evaluate mRNA levels of more pigmentation genes such as *MITF* and *TYR*. *MITF* is considered both a downstream target gene of MC1R signalling as well as a regulator of *MC1R* expression. Both *MITF* and *TYR* are expected to be expressed at a very low level in white and rust feathers.

Another open question is why there are expression differences of *MC1R* in white ornamental feathers versus white non-ornamental feathers and why both feather types do not show allelic imbalance. A low expression or loss-of protein function of MC1R is usually resulting in red pheomelanin. White however often seems to be the result of a lack of melanocytes (Thalmann *et al.*, 2017) or the presence of undifferentiated melanocytes (Lin *et al.*, 2013). Examining white growing feathers using different melanocyte maturation markers will possibly reveal different mechanisms in white ornamental and non-ornamental feathers as the latter ones seem to have a high expression of *MC1R* but still no pigment production at all. This suggests that the melanocytes are differentiated and pigment production might occur but that pigment transfer could be hampered. This could be the result of epigenetic modulation due to ageing, feather position or feather type. Immunohistochemistry and *in-situ* experiments can also provide opportunities to study additional factors such as ASIP and their spatial distribution within the feather follicle.

Repeating the *in-vitro* experiments using an avian cell line and differently/ non-tagged MC1R proteins appears to be of great importance to shed light on functional differences between sMC1R and iMC1R. Since gene expression and functional data are not coming together to a coherent story, chances are that different experimental conditions might lead to a different, more natural outcome. Our surface expression data is suggesting that the iMC1R is not efficiently transported to the membrane and that the great majority of it stays internalized. This is a rather unusual finding and it needs to be validated in other cells and with other constructs in order to ensure that the receptor is properly processed in the cell model. We are in fact currently in the process of repeating the experiments using an embryonic chicken fibroblast cell line as well as untagged receptors.

In a broader context it would be interesting to explore the purpose of the great pigmentation variety between and among independents and satellite males. Pigment production is considered to involve costs and must provide a fitness advantage in order to be maintained (Galvan and Solano, 2009). The pigment intensity variation between satellites and independents might make each morph more easily recognizable for each other and could facilitate the male setup on the leks ultimately attracting more females. For females in turn, it might be advantageous to assign males from the distance to a particular morph and make a decision on whether to visit a lek or not. However, since the colouration is not the only distinguishable characteristic between the morphs, it is possible that it is just a 'by-product' of the linkage of a variable set of genes into one 'supergene' (e.g. by creating a linkage to regulatory and missense mutations in *MC1R*).

In summary, the ruff provides a great platform to investigate various aspects of sexual selection as well as mate choice, and a unique opportunity to explore the molecular basis and evolution of complex traits such as plumage pigmentation, male ornaments and reproductive behaviour.

4 Conclusion

The research in this thesis provides important insights into the molecular complexity of pigmentation genetics and pathways in both domestic and wild birds.

In study I, we have identified a gene involved in within-feather pattern formation, which previously has only been known to determine pigment distribution across the avian body: *MC1R*. Although our data suggest that *MC1R* is indeed interacting with other genes to create a distinct pattern, we were able to contradict the longstanding idea that autosomal barring in the Fayoumi breed is always the result of a mandatory interaction between the two loci *Pg* and *Db*. Comparisons with other proposed *Pg*-carrying breeds suggest further that there is no distinct, shared *Pg* locus but that different alleles containing activating mutations in *MC1R* might be the key to creating the astonishing within-feather pigmentation variation observed in chicken. These findings provide a reminder that although knowledge collected through traditional Mendelian genetics is very valuable when mapping phenotypic traits, its implications on molecular functioning might be a bit more complex than initially predicted.

Study II is one of the few in depth investigations to unravel the molecular action of mutations associated with a specific within-feather pattern. Our results show that the occurrence of multiple mutations on one haplotype can create very defined patterns over time and reinforces the important mechanism of 'evolution of alleles' in creating complex pigmentation phenotypes, which otherwise might only be expected from interaction of two or multiple different genes. Furthermore the study supports previous findings, which suggested that melanocyte migration, proliferation and differentiation in the feather follicle provide key elements to melanin pattern formation on individual feathers. Both study I and II provide molecular clues on which the suitability of Alan

Touring's natural pattern formation theory to pigment pattern formation in birds can be further discussed.

In study III, we found a large inversion containing over 90 genes, which is determining not only alternate reproductive behaviour in ruff males, but also diverse physiological as well as plumage differences. Our findings support previous discoveries highlighting the importance of large-scale chromosomal rearrangements in evolution of complex traits under natural conditions and suggest a genetic mechanism how pigmentation can become part of complex behavioural traits when inherited together with other loci as a 'supergene'. With study III we have also paved the road for future research to investigate the role of genes involved in regulation of hormone metabolism and reproductive behaviour, which will provide a better understanding on how complex physiological changes e.g. during breeding season are mediated in birds.

In study IV, we tried to set out to understand the role of MC1R for pigmentation differences observed between two reproductive morphs in the ruff: the satellite and the independent. Although the research on *MC1R* in birds is plentiful, this is the first study attempting to understand plumage variation, which is not permanent but only displayed seasonally. Our findings suggest that both regulatory and missense mutations in this receptor provide a diverse basis for variable pigmentation in birds. This might be surprising as *MC1R* is a fairly short and simple gene with a rather well defined function as a membrane receptor and simplistic signalling pathway. Future research should be encouraged to understand how *MC1R* appears to hold the key to all sorts of pigmentation variation in birds: whole body colouring just as much as pigment patterning across the body or on individual feathers as well as for seasonal plumage development.

In summary the findings of this thesis contribute to a better understanding of the complexity of pigmentation variation in birds and provide a basis for future research.

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Popular science summary

Birds are the most colourful group of vertebrates on our planet. Their feathers exhibit an impressive range of both – dull colours such as white to brown, grey and black – as well as vibrant pink, red, yellow or blue and green in different shades and often topped with a fine iridescent shimmer depending on the angle of light entering the feather. Pigmentation can be uniform across the entire body or occur in patches, stripes and dots on different body parts or even within individual feathers. Furthermore, colouration can vary in different life stages and between sexes, depending on season and availability of resources, hormonal and health status of an individual. However, very little is known about the genetic mechanisms underlying this astonishing plumage colour variation. This thesis is comprised of four studies, which shed more light on molecular processes and genetic variants responsible for colour pattern formation on individual feathers as well as across the body of two different bird species.

In study I and study II, we utilize the domestic chicken (*Gallus gallus domesticus*) as a model to understand two different types of within-feather barring patterns: autosomal and sex-linked barring. Autosomal barring is the trademark of the Fayoumi chicken breed and we generated an intercross by mating some birds from this breed with another breed (Light Brown Leghorn), which did not show any within-feather pattern. A subset of the female offspring (F_1) was crossed again with a Light Brown Leghorn male and the second generation of chicks was evaluated for their feather pattern appearance as well as genotyped with molecular methods. Our genomic analysis demonstrated that a defined region on chromosome 11 is harbouring the genetic variant that is the major cause for this specific barring pattern. The region contains about 29 genes, among them one, which is coding for a receptor located on the surface of pigment cells. This receptor is called Melanocortin 1 receptor (MC1R) and is known to be involved both in the production of dark pigment as well as pigment cell division and biology. We

found a mutation in the gene *MC1R* in chicken exhibiting autosomal barring, which is predicted to alter the function of the receptor. Furthermore our pedigree analysis suggested that other genes are affecting the clarity of the barring pattern. Chickens carrying the mutation in *MC1R* as well as a mutation on chromosome 1 close to a gene named *SRY - related HMG-box 10* (*SOX10)*, causing the Dark brown (Db) phenotype, developed a more clear barring patterns than those that did not carry the respective mutation on chromosome 1. This study is the first one specifically implicating *MC1R* in within-feather pattern formation in chickens and is contradicting the longstanding idea that autosomal barring is the result of a mandatory interaction of two loci on chromosome 1 named *Patterning* (*Pg*) and *Dark brown* (*SOX10*).

In study II, we performed molecular experiments to understand the consequences of four mutations, which have been implicated in sex-linked barring in chicken in a previous study. We found that two of the mutations increase the activity of the *cyclin-dependent kinase inhibitor 2A* (*CDKN2A*) in feathers and that this causes faint but clearly barred feathers. The two other mutations are located within the gene product of *CDKN2A* and hamper the interaction of the protein with another protein named mouse double minute 2 homolog (MDM2). Our findings suggest that only a combination of both – the higher gene activity together with either one of the two missense mutations in *CDKN2A,* are causing the pronounced and clear alteration of dark and white bars observed in typically sex-linked barred chicken breeds such as the Barred Plymouth Rock chicken.

In study III and IV, we have explored the plumage colour diversity observed in the ruff (*Philomachus pugnax*). Ruff males can belong to either of three reproductive strategies termed ‘independent’, ‘satellite’ or ‘faeder’, which differ in behavioural and physiological aspects as well as in their plumage appearance and coloration. We found that all satellites and faeders carry an inversion: a region of a chromosome, which broke off and was re-inserted in the opposite direction but in the same position of the same chromosome. This event has disrupted an important gene (*centromere protein N*, *CENPN*), of which all animals require at least one functioning copy. Satellites and faeders therefore always carry only one inverted chromosomal region while the second chromosome is normal (they are heterozygous for the inversion). The inverted region contains about 90 genes, some of them involved in hormone metabolism and plumage colouration. We found a number of additional genetic variants, which further distinguish the satellites and the faeders from the independent morph and from each other. We were able to estimate that the independent represents the ancestral state, while the faeder and satellite variant have evolved after the initial inversion happened

about four million years ago. This is one of the most spectacular examples of a balanced polymorphism that has been maintained for million of years.

During breeding season independent males exhibit an impressive variation in their plumage coloration around the neck (ruff) as well as on the head (head tufts). In contrast, satellite males do also develop a ruff and head tufts but these feathers tend to be less variable in colour, much lighter or even purely white. When we compared the DNA sequence of the *MC1R* gene present in satellites and independent males, we found four mutations that cause differences in the protein sequence between these two different gene variants. We first examined the gene activity of the two different *MC1R* gene variants (*I-Wild-type* and *S*– associated with the *satellite* chromosome and therefore the mutations) and found that the gene activity of the *I* variant was much higher than the *S* variant in coloured feathers of satellite males. In white feathers however, both gene variants were equally active. This suggests that mutations regulating the activity of *MC1R* are underlying the activity difference in coloured versus white feathers in satellite males. In the next step we transferred both gene variants into cultured cells in the lab and found that the *S* variant was causing a permanently (constitutively) active receptor, which resulted in higher intracellular signalling than the *I* variant. This was an unexpected finding because the *S* variant is linked to very light breeding plumage. We therefore had expected a lower signalling ability of the receptor resulting from this *MC1R* gene variant. Our results show that there are clear functional differences between the *S* and the *I* gene variant for *MC1R* and that this probably contributes to the very striking differences in breeding plumage between those males, although they are undistinguishable from each other outside the breeding season.

Populärvetenskaplig sammanfattning

Fåglarnas häpnadsväckande variation i färgteckning överträffar färgvariationen hos alla andra ryggradsdjur. Fjäderdräkten uppvisar en förbluffande variation av matta färger från vita till bruna, gråa till svarta samt briljanta nyanser av rosa, rött, blått och grönt ofta kombinerade med skimrande färger som uppkommer när ljuset bryts i fjäderdräkten till exempel hos en skata. Färgteckningen kan vara enhetlig över hela kroppen eller bestå av fält med olika färger och det kan dessutom förekomma fläckar, band och andra mönster som ökar komplexiteten. Dessutom kan färgteckningen variera under individens utveckling, mellan könen, mellan olika årstider samt beroende på näringsstatus och hälsotillstånd. Kunskapen om de genetiska mekanismer som styr fåglars variation i färgteckning är dock bristfällig. Denna avhandling som består av fyra delarbeten sprider nytt ljus på de molekylära och genetiska mekanismer som påverkar färgmönster i enskilda fjädrar såväl som färgteckningen hos två olika fågelarter, tamhönan och brushane.

I arbete I och II så har vi använt tamhönan (*Gallus gallus domesticus*) som en modell för att studera två olika typer av vattrade fjädrar (fjädrar med tydliga ränder med olika intensitet i pigmenteringen), den ena med dominant autosomal nedärvning och den andra med dominant könsbunden nedärvning. Autosomal vattring är en karaktäristisk färgteckning hos bland annat Fayoumi som är en egyptisk hönsras. Vi korsade Fayoumi höns med en tupp av rasen Light Brown Leghorn som saknar genvarianten för autosomal vattring. F_1 hönsen återkorsades därefter med en Light Brown Leghorn tupp och avkommorna från denna korsning användes för att undersöka den genetiska bakgrunden till autosomal vattring genom noggrann registrering av färgteckning samt genetisk analys. Våra analyser visade att en region på hönsens kromosom 11 har ett avgörande inflytande på förekomsten av autosomal vattring. Denna kromosomregion innehåller 29 gener men en gen stod ut som en uppenbar kandidatgen för denna variation, *melanocortin 1 receptor* (*MC1R*) eftersom den har en mycket väl etablerad roll att reglera

pigmentering hos fåglar och andra ryggradsdjur. Vi fann en mutation i *MC1R* kopplad till autosomal vattring som förväntas ha en tydlig effekt på denna receptors funktion. Vi kunde också visa att en genvariant (*Dark brown*) i *SRY-related HMG-box 10 (SOX10*) genen påverkade hur tydlig vattringen blev. Detta är den första studien som specifikt visar att genetisk variation i *MC1R* kan påverka hur mönster uppkommer i enskilda fjädrar. Studien motsäger också tidigare studier som har indikerat att autosomal vattring beror på två olika genvarianter på kromosom 1 *Dark brown* (*SOX10*) och *Patterning* (*Pg*).

I arbete II genomförde vi olika molekylära analyser för att undersöka vilken betydelse fyra tidigare beskrivna mutationer har för uppkomsten av könsbunden vattring, som kallas gökfärg på svenska eftersom fjäderteckningen påminner om gökens vattrade fjäderdräkt. Vi kunde visa att två icke-kodande mutationer ökade genuttrycket av en tumörsuppressorgenen *CDKN2A* (*cyclin-dependent kinase inhibitor 2A*) specifikt under fjäderns utveckling. Höns som bara bär dessa icke-kodande mutationer får en urblekt, nästan vit fjäderdräkt, med svag vattring. De två andra mutationerna är kodande och förändrar proteinsekvensen av *CDKN2A* och dessa hämmar interaktionen mellan detta protein och ett annat protein mouse double minute 2 homolog (MDM2). Våra resultat visar att kombinationen av dessa regulatoriska mutationer samt en av de kodande mutationerna krävs för uppkomsten av gökfärg, med mycket skarpa kontraster mellan vita och mörka band på fjädern. Denna spektakulära färgteckning finns hos bland annat höns av rasen Barred Plymouth Rock.

I arbete III och IV så har vi undersökt variation i fjäderdräkten hos brushanen (*Philomachus pugnax*). Det finns tre olika typer (morfer) av hannar (oberoende, satellit och faeder) hos denna art. Dessa har tydligt olika reproduktiva strategier och de skiljer sig åt med avseende på beteende, storlek och fjäderteckning. Vi fann att satellit och faeder hannar bär på en inversion, det vill säga en kromosomregion som snurrat runt 180 grader jämfört med ursprungsvarianten som de oberoende hannarna har. En av inversionsbrytpunkterna bryter upp och inaktiverar en essentiell gen *CENPN* (*centromere protein N*). Det innebär att inversionen är letal i homozygot form (dubbla kopior) och alla satellit och faeder hannar bär på inversionen på den ena kromosomen och vildtypsvarianten på den andra, det vill säga de är heterozygota för inversionen. Regionen som omfattas av inversionen innehåller cirka 90 gener, vissa av dessa påverkar metabolismen av könshormoner och andra som *MC1R* påverkar fjäderdräkten. Vi kunde visa att inversionen uppkom för cirka 4 miljoner år sedan och att utvecklingen av brushanens komplexa parningsstrategi reflekterar en lång evolutionär process som säkerligen beror på genetiska förändringar i ganska många gener. Detta är ett

av de mest spektakulära exemplen inom biologin på en balanserad genetisk polymorfi som har upprätthållits i miljontals år.

Oberoende hannar uppvisar en häpnadsväckande variation i fjäderdräkten och har halskrage och huvudtofsar med starka färger under parningssäsongen. I kontrast mot detta så har satelliter en halskrage och huvudtofsar med ljusa eller vita färger. När vi jämförde DNA sekvensen hos *MC1R* genvarianten hos oberoende hannar och satellit hannar så fann vi fyra mutationer som orsakar skillnader i proteinsekvensen. I arbete IV har vi studerat den funktionella betydelsen av dessa mutationer för att utreda om denna genetiska variation är en viktig förklaring till skillnaden i fjäderdräkt mellan dessa typer av hannar. Först undersökte vi genuttrycket av dessa genvarianter (*I*=vild-typ och *S*=associerad med *satellit*-kromosomen) och fann att det relativa uttrycket av *I*-varianten var tydligt högre än *S*-varianten i fjädrar med tydlig pigmentering medan det inte förelåg någon skillnad i de vita fjädrarna. Detta visar att regulatoriska mutationer ligger bakom denna skillnad i genaktivitet i olika typer av fjädrar. Därefter uttryckte vi *I* och *S* varianterna av *MC1R* i odlade celler och fann att *S* varianten är konstitutivt aktiv (på hela tiden) och resulterar i en högre intracellulär signalering jämfört med *I* varianten. Detta var ett oväntat fynd eftersom *S* varianten är kopplad till en mycket ljus fjäderdräkt under parningssäsongen och vi hade förväntat oss en genvariant som var mindre aktiv. Våra resultat visar att det föreligger tydliga funktionella skillnader mellan *S* och *I* varianten för *MC1R* och att detta sannolikt bidrar till den mycket slående skillnaden i fjäderdräkt mellan dessa hannar under parningssäsongen trots att de knappast visar några skillnader under resten av året.

Résumé vulgarisé intégral

Les oiseaux sont parmi les vertébrés les plus colorés de notre planète. Leurs plumes présentent une gamme impressionnante de couleurs ternes (blanc à brun, gris et noir) ou vives, telles qu'un rose vif, rouge, jaune ou bleu et vert dans différentes nuances, souvent couronnées d'un brillant irisé selon l'angle de lumière entrant dans la plume. La pigmentation peut être uniforme sur tout le corps ou apparaitre sous forme de taches, de rayures et de points sur différentes parties du corps ou même à l'intérieur de plumes individuelles. De plus, la coloration peut varier selon les stades de la vie et les sexes, selon la saison et la disponibilité des ressources, l'état hormonal et l'état de santé d'un individu. Malheureusement, on sait très peu de choses sur les mécanismes génétiques qui sous-tendent cette étonnante variation de couleur du plumage. Cette thèse est composée de quatre études, qui éclairent davantage les processus moléculaires et les variants génétiques responsables de la formation de motifs colorés sur les plumes individuelles ainsi que sur le corps de deux espèces d'oiseaux.

Dans l'étude I et l'étude II, nous utilisons le poulet domestique (*Gallus gallus domesticus*) comme espèce modèle pour comprendre deux types différents de rayures à l'intérieur des plumes: la barrure autosomale et la barrure liée au sexe. La barrure autosomale est une caractéristique de la race de poule Fayoumi, dont nous avons croisé quelques individus avec une autre race (Leghorn dorée), qui ne montre pas de dessins sur les plumes. Les descendants F_1 ont été croisés en retour avec un mâle Leghorn dorée et la génération de poussins obtenus a été décrite pour le dessin de plumage et étudiée par séquençage et génotypage. Notre analyse génomique suggère qu'une région particulière du chromosome 11 abrite le variant génétique responsable de ce type particulier de barrure. Cette région contient environ 29 gènes, dont un gène codant pour un récepteur spécifique aux cellules pigmentaires, appelé récepteur à la *mélanocortine 1* (*MC1R*), connu pour être impliqué dans la production de pigment foncé, comme dans la division et la biologie des cellules

pigmentaires. Nous avons identifié une mutation de *MC1R* chez des poulets présentant la barrure autosomale, qui est capable d'altérer la fonction du récepteur. De plus, notre analyse du pedigree suggère que d'autres gènes affectent la clarté du motif rayé. La présence simultanée de la mutation dans *MC1R* et d'une délétion sur le chromosome 1 proche du gène *SRY - apparenté HMG-box 10* (*SOX10*), précédemment associée au phénotype 'brun foncé (locus *Db*), améliore la précision du dessin de plumage par rapport au phénotype des animaux ne portant pas la délétion. Cette étude est la première à impliquer spécifiquement le gène *MC1R* dans la formation de motifs à l'intérieur des plumes chez les poulets, et contredit l'idée de longue date selon laquelle la barrure autosomale résulte d'une interaction obligatoire entre deux locus du chromosome 1, nommés *Patterning* (*Pg*) et *Dark brown* (*SOX10*).

Dans l'étude II, nous avons effectué des expériences moléculaires pour comprendre les conséquences de quatre mutations précédemment impliquées dans la barrure liée au sexe chez le poulet. Nous avons constaté que deux des mutations augmentent l'activité de l'inhibiteur de *kinase 2A cyclinodépendant* (*CDKN2A*) dans les plumes, ce qui conduit déjà à des plumes faiblement pigmentées mais clairement barrées. Les deux autres mutations sont localisées dans la protéine codée par *CDKN2A* et entravent son interaction avec une autre protéine nommée double-minute-2 homologue de la souris (MDM2). Nos résultats suggèrent que seule une combinaison des deux types de mutation- les mutations régulatrices en amont et l'une ou l'autre des deux mutations non-sens de *CDKN2A*- provoque l'alternance prononcée et nette des barres foncées et blanches observées chez les races de poulets montrant une barrure liée au sexe caractéristique, comme chez le poulet 'Barred Plymouth Rock'.

Dans les études III et IV, nous avons exploré la diversité de couleur du plumage observée chez le chevalier combattant (*Philomachus pugnax*) qui présente trois formes reproductives chez le mâle, dénommées 'indépendant', 'satellite' et 'faeder'. Ces morphotypes diffèrent par des aspects comportementaux et physiologiques ainsi que par la coloration de leur plumage. Nous avons constaté que tous les 'satellite' et les 'faeder' portent une inversion chromosomique - une région d'un chromosome qui s'est détachée et a été réinsérée dans la direction opposée mais dans la même position du même chromosome. Cet événement a perturbé un gène important (codant pour la protéine centromère N, CENPN), dont tous les animaux ont besoin d'au moins une copie fonctionnelle. Les 'satellite' et les 'faeder' ne portent donc toujours qu'une seule région chromosomique inversée alors que le deuxième chromosome est normal (ils sont hétérozygotes pour l'inversion). La région inversée contient environ 90 gènes, dont certains sont impliqués dans le

métabolisme hormonal et la coloration du plumage. Nous avons trouvé un certain nombre d'autres variants génétiques qui distinguent le ‘satellite’ et le ‘faeder’ entre eux et vis-à-vis du type ‘indépendant’. Nous avons pu estimer que le type ‘indépendant’ représente l'état ancestral, tandis que les formes variantes ‘faeder’ et ‘satellite’ sont apparues successivement et plus tard dans le temps.

Dans l’étude IV, nous nous sommes ensuite intéressés à quatre mutations localisées dans le gène *MC1R* du type ‘satellite’ pour explorer plus en profondeur leurs conséquences fonctionnelles. Nous avons constaté que le variant du gène *MC1R* situé sur le chromosome inversé du type ‘satellite’ était moins exprimé que celui du chromosome non inversé. Cependant, au total, le gène *MC1R* était plus exprimé dans les plumes des ‘satellite’ que dans les plumes des ‘indépendant’ de la même couleur. Il s'agit d'une constatation inattendue étant donné que la coloration du plumage des mâles ‘satellite’ est beaucoup moins intense que celle des ‘indépendants’ et qu'on s'attendait à ce qu'elle soit associée à une activité de *MC1R* plus faible. Nous avons également constaté en culture cellulaire que la signalisation induite par le récepteur MC1R du type ‘satellite’ dans la membrane cellulaire diffère de celle du récepteur de type ‘indépendant’, et n’est pas affectée de la même façon par des mécanismes tels que le transport à la membrane cellulaire. Nos résultats suggèrent que toutes ou certaines des quatre mutations du gène *MC1R* du type ‘satellite ‘ modifient sa fonction et pourraient contribuer à la variation de couleur de plumage observée entre les mâles de type ‘satellite’ et les mâles de type ‘indépendant’ pendant la saison de reproduction.

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Title : Molecular identification of colour pattern genes in birds

Keywords : birds, chicken, ruff, pigmentation, plumage, feather pattern, *MC1R*, autosomal barring, sex-linked barring, melanocytes, feather follicle

Abstract : Birds display a spectacular range of plumage pigmentation. The purpose of this thesis was to elucidate genetic mechanisms that contribute to pattern formation on individual feathers and the body.
In study I and II, we investigated two barring patterns in chicken. We show that in the Fayoumi breed autosomal barring is associated with a 1Mb un-recombined region on chromosome 11, which contains the *MC1R* gene. Our functional analysis strongly suggests that autosomal barring is primarily caused by activating *MC1R* mutations and that other loci contribute to the appearance of the pattern. In study II, we demonstrate that sex-linked barring is created by a combination of *cis*-regulatory and missense mutations in the *CDKN2A/* ARF gene. We demonstrate that the up-regulation of *CDKN2A* expression is caused by non-coding mutation(s) and is resulting in a dilute barring pattern. Functional testing revealed that the two missense mutations in ARF hamper its function and restrict the diluting effect of the non-coding mutations. Only the combination of both regulatory and missense mutations generates clear barring pattern as observed e.g. in the Barred Plymouth Rock. In study III and IV, we investigated the genetic mechanisms driving pigment pattern variation in the ruff (*Philomachus pugnax)*. We first identified a 4.5 Mb inversion to be associated with the two male reproductive morphs called satellite and faeder. These morphs differ substantially in behavior, reproductive strategy, body size and plumage appearance between each other as well as from the third, more prevalent morph, the independent. The inversion disrupts the *CENPN* gene making this genomic re-arrangement homozygous lethal. We identified a large set of variants; among them four missense mutations in *MC1R* associated with the *Satellite* allele. In study IV, we explored whether these *MC1R* mutations are contributing to the light display plumage of the satellite morph. Our data shows that *MC1R* is up-regulated in all coloured satellite feathers and that this is due to a higher expression of the *Independent* allele. Evaluation of MC1R signaling in cell culture models subsequently revealed that the mutations alter receptor properties such as cAMP production, sensitization and surface expression but also suggests that transfection assays using mammalian cells might not reveal the complex function MC1R is most likely having in avian melanocytes.

Titre : **Identification de gènes de dessin de plumage chez les oiseaux**

Mots-clés : oiseaux, poulet, chevalier combattant, coloration du plumage, dessin de plumage, MC1R, barrure autosomale, barrure liée au sexe, mélanocyte, follicule plumeux

Résumé : Le plumage des oiseaux montre une diversité de coloration spectaculaire. L'objectif de cette thèse est d'élucider les mécanismes génétiques contribuant à cette diversité en étudiant plus particulièrement la formation de dessins sur la plume et sur le corps. Les études I et II sont consacrées à deux dessins de barrure chez le poulet : la barrure autosomale et la barrure liée au sexe. En réalisant un croisement en retour à partir de la race Fayoumi, nous avons pu démontrer que la barrure autosomale est associée à une région du chromosome 11 et porte le gène de pigmentation *MC1R*. L'analyse fonctionnelle met en évidence qu'une mutation faux-sens, et non une modification de régulation, a le rôle d'une mutation causale. Dans l'étude II, nous démontrons que la barrure liée au sexe est créée par la combinaison de mutations de régulation en cis et de mutations faux-sens dans le gène *CDKN2A*. L'activation de l'expression de *CDKN2A* par deux mutations non-codantes détermine un phénotype de barrure très dilué. Des tests fonctionnels *in vitro* révèlent que les deux mutations faux-sens identifiées dans le même gène restreignent sa fonction et diminuent l'effet de dilution des mutations non-codantes. Seule la combinaison des mutations de régulation et d'une des mutation faux-sens, produit la barrure bien nette observée dans les races de poule actuelles. Les études III et IV précisent les mécanismes contrôlant la variation des dessins de plumage chez les oiseaux sauvages. Le chevalier combattant *Philomachus pugnax* présente trois formes reproductives chez le mâle, dénommées 'indépendant', 'satellite' et 'faeder'. Nous avons d'abord identifié une inversion de 4.5 Mb chez les mâles satellite et faeder, qui diffèrent nettement entre eux, comme de l'indépendant, par leur comportement, leur stratégie reproductive et l'aspect de leur plumage. Un examen plus précis de la structure de l'inversion a montré que les points de cassure interrompaient le gène *CENPN* ce qui rend l'inversion létale à l'état homozygote. Nous avons identifié un grand nombre de variants contribuant assez probablement au phénotype et avons découvert quatre SNPs dans l'allèle *MC1R* du mâle satellite. L'étude IV a consisté à analyser la contribution de ces mutations de *MC1R* au plumage clair du mâle satellite pendant la reproduction. Nos données montrent que *MC1R* est activé dans les plumes colorées du mâle satellite en raison d'une plus forte expression de l'allèle *indépendant*. L'étude de la signalisation de MC1R en culture cellulaire a ensuite révélé que les mutations altèrent les propriétés du récepteur, mais suggère aussi que l'évaluation fonctionnelle du MC1R aviaire en cellules mammaliennes ne rend pas complètement compte du rôle complexe que *MC1R* joue dans les mélanocytes aviaires.