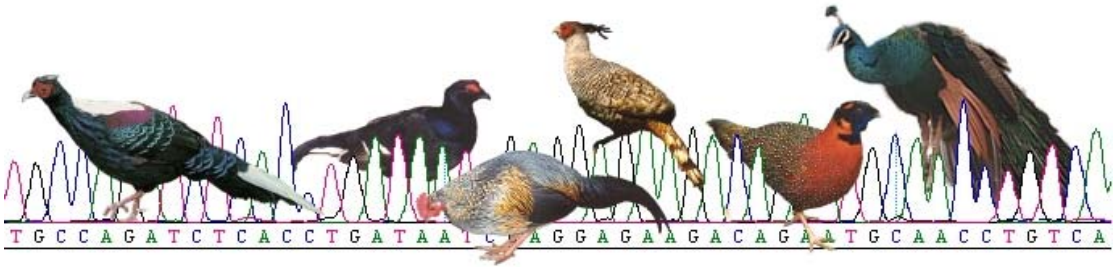
# The Evolutionary Genetics of Sexually Selected Plumage Colour Traits in the Galliform Birds

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**2006**

This dissertation is submitted for the degree of Doctor of Philosophy

## Declaration

This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and acknowledgements.

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## Summary

Extravagant male plumage traits in birds are a classic example of sexual selection. However we know very little about the units that selection is acting upon, the genes themselves – what are they and how are they influenced by sexual selection? In this study I focused on in the evolution and genetics of colouration the galliform birds. Several novel loci were used to create a well resolved phylogeny of this group. This was then used to investigate and reconstruct the evolution of sexual plumage dichromatism. Four pigmentation genes were sequenced in an array of galliform species. A measure of the rate of evolutionary change (dN/dS) at these loci was then compared between lineages with different strengths of sexual selection, using sexual dichromatism as the main index of sexual selection. I found evidence for sexual selection acting at the *MC1R* locus, in the form of a robust correlation between dN/dS and sexual plumage dichromatism that was not found at any of the other loci. I then went on to investigate the evolution and population genetics of *MC1R* in the grouse, focusing on the strongly dichromatic black grouse and the relatively monochromatic red grouse. I found some evidence for an adaptive change at this locus between these species. Finally I used a candidate gene approach to investigate the role of several genes in avian pigmentation using the Japanese quail (*Coturnix japonica*) as a model system. I found evidence that the avian *agouti* gene is involved in dorso-ventral pigmentation patterning and a regulatory mutation at this locus that produces a yellow phenotype. In addition point mutations at *MC1R* and *TYRP1* were found to be responsible for producing pigmentation variants. I then compared the expression of several of these candidate genes in male and female common pheasants (*Phasianus colchicus*) and found lower *TYRP1* expression in males. Knowledge of the genetic basis of secondary sexual traits and the action of sexual selection at this level could have important implications for our understanding of the process of sexual selection as a whole.

## Acknowledgements

I would firstly like to thank my supervisor, Nick Mundy, who gave invaluable help, advice and support at all stages of this project and gave useful comments on this manuscript. Terry Burke conceived some of the original ideas on which this project was based and also gave many helpful comments during its development.

This work would not have been possible without the many people and organisations that donated samples. The samples for the comparative work were kindly donated by: Andrew Kitchener of the National Museum of Scotland; Stuart Wilson and John Corder of the World Pheasant Association; Joanne Cooper of the Natural History Museum Bird Group, Tring; Claire Spottiswoode of the University of Cambridge; Malcolm Brockless and David Baines of the Game Conservancy Trust; and Dada Gottelli of the Zoological Society of London. In addition, John Corder generously allowed me to use his collection of pheasant photographs. The red grouse samples were donated by Paul Johnson of the University of Glasgow, and the black grouse samples by Rauno Alatalo of the University of Jyväskylä. The quail samples came from the Institut National de la Recherche Agronomique (INRA), breeding facility at Nouzilly, France. I thank David Gourichon and Chantal Moussu for taking care of the experimental quails and collecting samples. I am also particularly grateful to Francis Minvielle for co-ordinating the breeding and sampling of the quails, for providing me with photographs of these birds and for his salient advice on possible candidate genes.

Many other people contributed to this project by providing their expertise, advice and participation in many fruitful discussions. I thank Ian Owens for his advice on the comparative analysis and Ally Phillimore for help with “Continuous”. Rebecca Kimball gave advice on cytochrome B sequencing. I would like to thank Adrian Friday, Nick Davies and Claire Spottiswoode in the Cambridge Zoology department for their insightfull advice and comments. Michèle Tixier-Bouchard also gave helpful comments on this work and advice on candidate gene selection. I also thank my examiners, Mike Brook and Staffan Bensch, for their many helpful comments. Sarah Johns, at Sheffield University, provided generous help and advice on RNA extraction and purification techniques and RT-PCR. John Chittock, in Sheffield, performed the checks for integrity of my RNA samples. I thank Tammie MacFie, Jenny Pastorini, Brenda Bradley and Marie Pointer for their help in the lab.

Finally I would like to thank my family and friends: Joan, Yvan, Valerie, Katie, Dean and Ed, for their continued support and encouragement. I am particularly grateful to Andrew Smith for his unfaltering support, computer and programming expertise, invaluable comments on this manuscript and other contributions too numerous to mention.

This work was funded by the NERC under the Environmental Genomics programme, grant number: NER/T/S/2002/00020.

## Dedication

For Phil and the galliform birds.

*"What a contrast is presented between the sexes by the polygamous peacock or pheasant, and the monogamous guinea-fowl or partridge!"*
Charles Darwin (1871)

# Contents

# Figures and Tables

# Chapter 1

# Introduction

## Summary

**Avian plumage traits are a classic example of sexual selection. Comparative and behavioural studies have shed some light on the function of sexually dimorphic plumage colour, which in most cases is now widely accepted to be the result of sexual selection. Sexual selection theory has progressed rapidly in recent decades with many models now being formulated based on quantitative genetic parameters. Empirical quantitative genetic studies have begun to test some of these models, but have tended to find strong environmental effects on the traits, preferences and fitness correlations. A molecular genetic approach would complement these studies by providing information about the loci involved and historical patterns of selection at these loci. We now have some understanding of the developmental mechanisms underlying the production of the complex integumentary structures that are feathers. In addition genetic and developmental studies have also improved our understanding of the process of melanin pigment synthesis and deposition.**

## Introduction

Ever since Darwin first outlined his theory of sexual selection (Darwin 1871), one of the most widely studied examples has been avian plumage traits (Andersson 1994). Plumage colour in particular has attracted a great deal of attention. A wide range of male plumage colours has been found to be used by females in mate choice (Petrie et al. 1991; Zuk et al. 1992; Omland 1996; Andersson et al. 1998; MacDougall and Montgomerie 2003) and in many cases these plumage colour traits have also found to act as condition-dependent indicators of male condition or viability (Hill et al. 1994; Veiga and Puerta 1996; Keyser and Hill 1999; Hill 2000; Keyser and Hill 2000; Møller and Cuervo 2003; Hill et al. 2005).

Sexual selection theory has largely focused on the ability of females to gain indirect (genetic) benefits by choosing males with ornamental traits (Andersson 1994), particularly in cases where males provide no care and females only obtain fertilisations (Kirkpatrick and Ryan 1991). However, a key assumption of these models is the presence of heritable genetic variation in these traits, which several quantitative genetic studies have failed to find (Griffith et al. 1999a; Hadfield et al. 2006). Other studies have found heritable variation in sexually selected traits but only under certain environmental conditions (Qvarnström 1999a). Molecular genetic studies may improve our understanding of the process of sexual selection, by allowing investigation of the units that sexual selection is acting upon, without the noise generated by environmental variation and by detecting historical patterns of selection.

Our understanding of the genetics of feather colour is primarily based on the genetics of mammalian hair colour (Jackson 1994). Feathers clearly differ structurally from hairs and can be patterned in two dimensions, rather than just one in hairs (Prum and Williamson 2002). Additionally, mammalian hair colour is due only to melanin,

whereas feathers can be coloured by pigments other than melanins and may also have nanometre scale structures that create colour by scattering light (Hill and McGraw 2006). However, melanins are still the main pigments used in feathers and our understanding of the genetic control of melanin deposition provides a basis for studies of avian feather colour at the molecular level (Mundy 2005). Studies of avian pigmentation genetics have primarily focused on the domestic fowl as they provide well-studied pigmentation variants (Cheng and Kimura 1990; Smyth 1990) and a range of powerful genetic resources (Consortium 2004). Our knowledge of the pigmentation genetics of chickens and quails can easily be extended to related birds in the order Galliformes, which include some of the classic examples of sexually selected plumage traits (Petrie et al. 1991; Zuk et al. 1992).

In this thesis, I attempt to characterise at the molecular level genes controlling melanic feather colour in the galliform birds. I also investigate the potential involvement of some of these genes in the production of sexually dichromatic plumage and look for signatures of sexual selection at the molecular level. In this introductory chapter, I discuss sexual selection theory and the possibilities for studying sexual selection at the level of the gene, before going on to outline our current understanding of the genetic control of feather colour.

## Sexual selection and plumage colour evolution

### The evolution and function of sexual dichromatism

Sexual dichromatism refers to the situation in which males and females differ in colour. In most species exhibiting sexual dichromatism generally the males are brighter, particularly during the breeding season. In order for this to occur, selection (natural or sexual) must be acting differently on the sexes and there must be a mechanism for bringing about sex-limited expression. It is expected that plumage colour will generally be correlated between the sexes. Only if genetic variation in sexual dimorphism arises through mutation or recombination will the sexes be able to evolve different optimal phenotypes (Lande 1980). Hormones control sex-limited expression of plumage traits in most birds (Kimball 2006). In the more basal orders of birds (the Struthioniformes, Galliformes and Anseriformes) plumage dichromatism appears to be largely oestrogen-dependent (Kimball and Ligon 1999); males have the "default" colouration and dichromatism is due to the presence of oestrogen in females. Kimball and Ligon (1999) suggested that this means that the most parsimonious reconstruction of plumage brightness is that both males and females of the early avian ancestor were brightly coloured, and dichromatism evolved through the loss of bright plumage by females (figure 1.1). However this reconstruction does not take into account variation in the level of "brightness". Genetic models of the evolution of sexual dimorphism suggest that prior to the evolution of sex-limited expression the phenotype will be a compromise between the selective pressures acting on the males and females. Once sex-limited expression is established both males and females will evolve simultaneously to become brighter and duller respectively (Lande 1980).

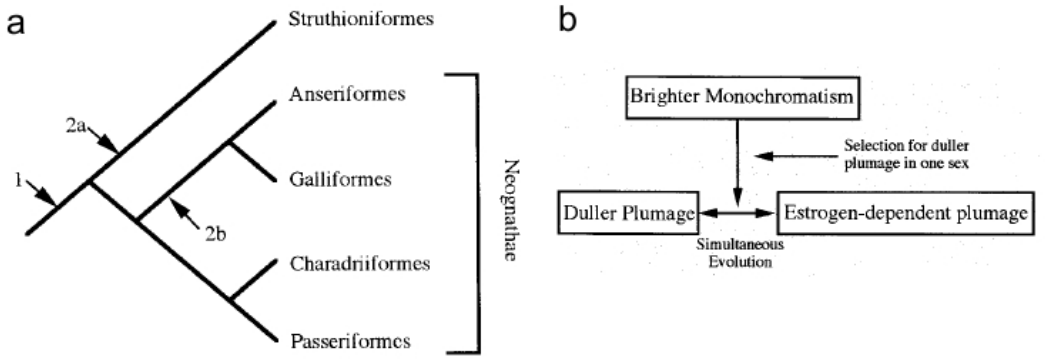



Figure1.1 The evolution of dichromatism within the birds. **a**, possible origins of oestrogen dependent dichromatism assuming a single origin (arrow 1) or 2 independent origins (arrows 2a and 2b). **b**, the most parsimonious reconstruction of the evolution of dichromatism within the Aves according to Kimball and Ligon (1999).

It does not appear to be the case, however, that bright female plumage traits are normally the result of maladaptive correlations between the sexes (Amundsen 2000). Transitions between dimorphism and monomorphism or vice versa appear to occur frequently in most birds (Price and Birch 1996; Omland 1997), suggesting that the transition is not difficult and unlikely to act as a constraint. Studies in new-world blackbirds and tanagers suggest that in these groups changes in female colour are more often responsible for changes in dichromatism than changes in male colour, implying more frequent change in the selective pressures acting on females perhaps due to social or sexual selection acting on female colour (Irwin 1994; Burns 1998). Evidence from other bird species also suggests that female ornamentation can function in mutual mate choice (Roulin et al. 2002; Kraaijeveld 2003).

Although sexual dichromatism is widely accepted to be due to the process of sexual selection (Sorci et al. 1998; McLain et al. 1999; Prinzing et al. 2002; Morrow and Pitcher 2003) alternative hypotheses have been proposed. A major one is the "unprofitable prey" hypothesis (Baker and Parker 1979), which suggests that male birds are more brightly coloured than females in order to warn predators that they are

either more distasteful or harder to catch. However many of the arguments put forward by Baker and Parker (1979) in their outline of this hypothesis are also consistent with sexual selection, for example higher conspicuousness among lekking and displaying males, and lack of paternal care. Experimental tests of this hypothesis have produced mixed results with suggestions that pied flycatcher males may suffer reduced predation compared to the cryptic females (Gotmark 1992), whereas conspicuous males in other species appear to suffer higher predation (Gotmark 1993; Montgomerie et al. 2001). Several aspects of avian life history also seem more consistent with bright male plumage being involved in intraspecific rather than interspecific signalling. For example, the time of moult into breeding plumage is generally associated with the timing of mate choice, not highest predation (Andersson 1994).

A further explanation for the occurrence of sexual dichromatism is differences in ecology between the sexes if, for example, they feed or roost in different areas. There seems to be very little evidence for this, except in some isolated cases, for instance some cavity-nesting females that are not exposed to visual predation (Heinsohn et al. 2005).

It therefore appears that in most cases sexual dichromatism can be largely attributed to sexual selection. Traditionally there were thought to be two ways in which sexual selection could act: either through female choice or male-male competition for females (Darwin 1871). However recently these two classifications have been broadened into two forms of mate choice, direct and indirect (Wiley and Poston 1996), highlighting that in most cases they are likely to be part of the same process of selecting the fittest or most viable males (Wong and Candolin 2005). Even in the most extreme cases, such as those involving sexual conflict, where males cause

direct harm to females, females may still obtain indirect benefits through the production of more competitive sons (Moore and Pizzari 2005). Although dichromatism is largely thought to evolve in response to female choice (Andersson 1994), there is evidence that in certain birds colour can be used as a badge of status in competitive interactions (Part and Qvarnström 1997).

Recent comparative studies have found associations between sexual dichromatism and mating system in birds (Figuerola and Green 2000; Dunn et al. 2001), suggesting that plumage colour is likely to play a role in sexual selection. Other studies have failed to find this association, including one by Höglund (1989), which found no association between lek-breeding and dichromatism. Bennett and Owens (2002) did not find an association between overall dichromatism and social mating system in a wide survey of avian taxa but did find an association with the level of extra-bond paternity, suggesting that plumage colour may be evolving in response to cryptic mate choice. When plumage colour was divided into its constituents: structural colours, melanins and carotenoids; the association with extra-bond paternity was found to be due to structural colours, while melanins were associated with sex-bias in brood defence (Owens and Hartley 1998; Bennett and Owens 2002). Carotenoids were not associated with any of the parameters of mate-choice, although this may be due to the restricted phylogenetic distribution of carotenoid-based colouration in birds, which was linked to availability of carotenoids in the diet. In contrast when considering only the North American passerines carotenoids appear to be correlated with sexual dichromatism, suggesting that in this group sexual selection may have promoted the use of carotenoids perhaps at the expense of melanins and structural colours (Gray 1996; Badyaev and Hill 2000).

**Sexual selection theory**

The process of sexual selection can broadly be divided into three phases: the origin of male ornaments and their association with improved reproductive success; the elaboration of male ornaments; and the maintenance of genetic variation in those ornaments in the face of strong directional selection. Several hypotheses have been put forward to explain these processes, most of which have been presented as distinct alternatives, although many could act in a complementary fashion, especially as some are particularly suited to explaining one or more of the phases of the sexual selection process.

The sensory bias hypothesis suggests that male ornaments evolve to exploit pre-existing female preferences that are present in their sensory systems due to natural selection on other traits or behaviours (Basolo 1990). Also known as sensory drive, this has largely been used to explain the origin of female preferences for male ornaments (Kokko et al. 2003; Arnqvist 2006). However, it has also been proposed as an alternative to other models of sexual selection and a quantitative genetic model suggests that this could function as a process of sexual selection with only natural selection acting on female preferences (Fuller et al. 2005). In other cases it has been suggested as a starting point for a sexual conflict process (Arnqvist 2006), as in the chase-away hypothesis (Holland and Rice 1998). Under this scenario male exploitation of female sensory biases causes females to mate suboptimally resulting in the evolution of female resistance. This could then lead to evolutionary cycles of male exploitation and female resistance and may result in the evolution of multiple ornaments only a small number of which would be functional in mate choice at any one time. The most convincing empirical examples of a chase-away type process come from invertebrates (Arnqvist 2006; Sakaluk et al. 2006). There are, however,

some avian examples where male traits appear to have evolved to exploit female sensory biases (Pryke and Andersson 2002; Madden and Tanner 2003). In addition to the pure sensory bias and chase-away hypotheses, there is also the possibility that traits arising as a result of sensory bias could evolve into honest condition-dependent signals (Garcia and Ramirez 2005).

Male ornaments could also arise de novo as indicators of male quality, either because they are genetically linked to a locus affecting male fitness or if they are condition-dependent. Females would then benefit from choosing males with those ornaments either directly, for example through improved access to resources, or indirectly through genetic benefits to their offspring (Darwin 1871; Fisher 1958). Zahavi (1975) proposed that the elaboration of males' ornaments due to their ability to reliably indicate male condition was the main or only process behind sexual selection. He suggested that ornaments act as handicaps to male survival thereby ensuring their honesty. This idea has since been elaborated and it has been shown that honest signals need not always be handicaps because higher quality individuals will be better able to afford the costs of associated with the signal (Getty 1998). Further models have suggested that a low level of "cheating" may be maintained in a population if signalling is on average honest (Johnstone and Grafen 1993) and even that in some situations signals need not be costly to be honest (Viljugrein 1997).

One problem with models invoking "good-genes" type indicator mechanisms, in which females select mates in order to obtain genetic fitness benefits for their offspring, is that they rely on the presence of additive genetic variance in fitness traits, which is generally not expected in populations at equilibrium (Taylor and Williams 1982; Kirkpatrick and Ryan 1991). Contrary to this expectation sexually selected traits are often highly variable within a species (Møller 1992) and, at least in some

cases, this variation appears to be genetically determined (Pomiankowski and Møller 1995). One possible resolution of this paradox is through the action of parasites (Hamilton and Zuk 1982). Due to the arms race between the immune system of the host and the defence system of the parasite, coadaptational cycles could maintain additive genetic variation in fitness in the population. This idea was extended further by the immunocompetence handicap hypothesis, which suggests that testosterone acts as a "double-edged sword" because it is required for the production of many secondary sexual ornaments and it also has a negative effect on the immune system (Folstad and Karter 1992). An alternative process for the maintenance of genetic variance was proposed in the "genic-capture" hypothesis (Rowe and Houle 1996). This proposes that costly secondary sexual traits will become condition-dependent and genetic variance in condition is high as it is controlled by a large number of loci and therefore acts as a large mutational target (Tomkins et al. 2004).

Perhaps one of the most important mechanisms in ornament elaboration is the "Fisherian" process (Fisher 1958). This relies on a genetic correlation between the male trait and female preference for that trait. The process may even be an inevitable effect of assortative mating, leading to linkage disequilibrium between the preference and trait loci, even if they are found on different chromosomes. This process has been modelled and shown to be sufficient to produce elaboration of male traits and female preferences (Lande 1981; Kirkpatrick 1982). However it can also function, and is perhaps most powerful, when combined with other processes such as "good-genes" or sexual conflict (Mead and Arnold 2004). An attractive feature of models incorporating a "Fisherian" process is that they seem best able to explain the diversity in secondary sexual traits seen across species. This is because small differences in

natural selection pressures, such as predation, between populations can lead to rapid divergence in ornaments and preferences (Pomiankowski and Iwasa 1998).

**The role of plumage colour in speciation and species recognition**

Species often differ most strongly in their secondary sexual traits, suggesting that these may evolve faster than other traits and may also drive speciation if they are involved in species recognition leading to prezygotic isolation (Young et al. 1994; Dale et al. 1999). As described above, some models of sexual selection do predict rapid evolution of male traits and female preferences that could lead to speciation (Lande 1981; Pomiankowski and Iwasa 1998). Further models have suggested that sexual selection could lead to sympatric speciation if there is sufficient variation in female preferences or if female preferences are frequency dependent (Higashi et al. 1999; Van Doorn et al. 2004). This process may occur even more readily if female preferences are not only genetically determined but also can be learnt, either through imprinting on the parental phenotype or through mate choice copying (Galef and White 1998; Price 1998; Swaddle et al. 2005). Consistent with this hypothesis many studies have found a correlation between the level of dichromatism or social mating system and species richness within a given taxa, suggesting that sexual selection may drive the speciation process (Barraclough et al. 1995; Mitra et al. 1996; Owens et al. 1999), although other studies have failed to find an association (Morrow et al. 2003).

The requirements of an ornament used in mate selection (particularly when this is condition-dependent) will not necessarily be same as those required for species recognition. Comparative studies by Bennett and Owens (2002) suggest that dichromatism in birds is better explained by sexual selection than species recognition. They did not find higher dichromatism in mainland when compared with island species, or sympatric as compared to allopatric species, as would be expected if its

primary function was species recognition. In contrast, divergence in plumage colour was best predicted by differences in signalling environment. In addition species-specific plumage characters were not more stereotyped (i.e. less variable) than other plumage characters as would be expected if they were used in species recognition. The opposite prediction of higher variability would apply for condition-dependent sexually selected traits. These conflicting requirements of species recognition and quality assessment on ornament variability would be expected to constrain each other if operating on the same ornament (Castellano and Cermelli 2006). However a model by Castellano and Cermelli (2006) suggests that traits could function in both recognition and selection if females have separate cognitive processes for recognition and comparison.

**The use of plumage colour in mate choice and male-male competition**

Many experimental studies have found that females use male plumage colour ornaments in mate choice (Petrie et al. 1991; Zuk et al. 1992; Omland 1996; Andersson et al. 1998; MacDougall and Montgomerie 2003). Other studies have found that sexually dichromatic plumage traits are not used by females (Beani and Dessi-Fulgheri 1995; Ligon et al. 1998; Hagelin 2001) and some have found that they are used instead in male-male competition (Mateos and Carranza 1997; Hagelin 2001). In many cases these findings may be related to the signalling potential of the male ornament in terms of its ability to honestly signal the quality of its owner. The classic studies by Petrie et al. demonstrated that not only did peahens prefer males with more eye-spots in their trains but also that these males suffered reduced predation and improved growth and survival (Petrie et al. 1991; Petrie 1992; Petrie 1994).

Different components of plumage colour ornaments may differ in their signalling potential. Carotenoids are thought to be good indicators of condition because they must be obtained in the diet and are also a required component of the immune system (Olson and Owens 1998). Studies of house finches (*Carpodacus mexicanus*) have suggested that carotenoid deposition in plumage is strongly dependent on diet and nutritional status (Hill et al. 1994; Hill 2000; MacDougall and Montgomerie 2003) and is mainly functional in mate choice, not social signalling (McGraw and Hill 2000a). In addition, coccidial infection in American goldfinches (*Carduelis tristis*) was found to affect carotenoid but not melanin-based plumage colouration (McGraw and Hill 2000b). Studies on great tits (*Parus major)* also found that nutrition had a larger effect on carotenoid-based than melanin-based ornaments, although fleas have produced the opposite effect (Fitze and Richner 2002; Senar et al. 2003). In contrast Kentucky warblers showed higher condition-dependence of melanin-based ornaments (Parker et al. 2003).

Melanin-based ornaments are often considered to be less costly than carotenoid-based colours because they are synthesised endogenously. However, this is not necessarily the case given that melanins are synthesised from the amino-acid tyrosine, which could be limited in the diet and pheomelanin additionally requires the amino-acid cysteine. It has also been suggested that the requirement of heavy metal ions by the tyrosinase enzymes could result in condition dependence (McGraw 2003). Studies of the melanin-based bibs of male house sparrows (*Passer domesticus*) have found mixed evidence for condition dependence of these ornaments. Some suggested that they may be condition-dependent at least in some circumstances (Møller et al. 1996; Veiga and Puerta 1996; Griffith 2000; Buchanan et al. 2003), while a direct test of the importance of dietary amino-acids failed to produce a response in ornament size

(Poston et al. 2005). Studies of these ornaments suggest that they function mainly in dominance interactions, not female choice, and that their honesty may be maintained by aggressive interactions giving them the label of "badges of status"(Møller 1988; Møller 1990; Griffith et al. 1999b; Vaclav and Hoi 2002; McGraw et al. 2003b). However, this type of signalling does not seem to be limited to melanic badges. The white forehead patch of collared flycatchers (*Ficedula albicollis*) also acts as a "badge of status" in addition to functioning in female choice (Part and Qvarnström 1997; Qvarnström 1999b). Similarly carotenoid-based patches are used to indicate dominance in the red-shouldered and red-collared widowbirds (*Euplectes axillaries* and *E. ardens*, respectively), rather than functioning in female choice (Pryke 2001; Andersson et al. 2002; Pryke and Andersson 2003).

Iridescent structural colours are widely used in displays, suggesting that they may have a role in mate choice or dominance interactions. The ultra violet (UV) component of structural plumage colour in blue tits (*Parus caeruleus*) is used in assortative mating and determining parental effort (Andersson et al. 1998; Limbourg et al. 2004). However the possibility that these structures are developmentally "self assembled" has been suggested to limit their potential for condition dependence (Prum 2006). In contrast several studies have found evidence for condition dependence of structural plumage colour mediated by nutritional condition (Andersson et al. 1998; Keyser and Hill 1999; McGraw et al. 2002; Siefferman and Hill 2005) or intestinal parasitic load (Doucet and Montgomerie 2003; Hill et al. 2005). Others have found evidence that males with stronger structural blue colours are of a higher quality (Keyser and Hill 2000; Siefferman and Hill 2003). These results come from both melanin-containing and non-melanin-containing structural colours (described in the later section on the production of structural colours) suggesting that

the presence or absence of melanin is not an important factor in determining condition dependence. How the condition dependence of these ornaments is maintained remains to be found. It is possible that they are costly to produce in ways that have yet to be elucidated or that they require high levels of maintenance to remain bright and so are costly in this way (Walther 2003; Walther and Clayton 2005).

## The evolutionary genetics of sexually selected traits

### Genetic models of sexual selection

The simplest models of sexual selection are based on a small number of loci. The classic model of Kirkpatrick (1982) assumed just 2 bi-allelic loci, one determining the female preference and one determining the male trait. In addition, for mathematical simplicity, most of these models assume haploid loci. These models predicted both stable (walk-towards) and unstable (runaway) equilibria in the frequency of male ornamentation and female choice (Kirkpatrick 1982; Seger 1985; Andersson 1994). Condition dependence can be added to these models via a third locus, which determines male condition (Pomiankowski 1987). These few-loci models all require a threshold level of female preference in order to produce any selection for the ornamentation allele at the male trait locus (figure1.2a). They are useful for predicting how a mutation, giving rise to a male ornamental trait, may spread after its origin in a population but do not predict how male ornaments that are based on multiple loci or multiple alleles at a single locus may evolve in response to sexual selection.

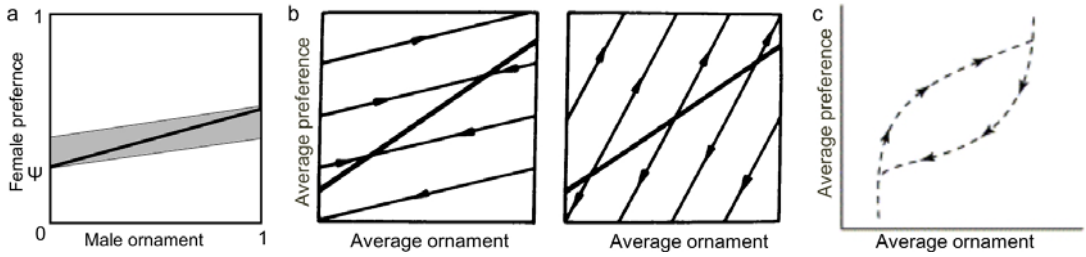



Figure1.2 Evolutionary pathways and equilibria in genetic models of sexual selection. **a**, Kirkpatrick's 1982 two-locus model of sexual selection. The solid line indicates the line of equilibria. Ψ indicates the threshold female preference required for selection on the male ornament. Deviations below the grey shaded area will lead to loss of the male ornament and above the shaded area will lead to fixation of the ornament (After Anderson, 1994). **b**, Lande's (1981) basic quantitative genetic model showing stable (walk-towards, left) and unstable (runaway, right) outcomes. The solid lines indicate equilibria and the arrowed lines the evolutionary trajectories. **c**, stable limit cycle of ornament evolution (from Mead and Arnold 2004).

Quantitative genetic models have been developed in order to answer these questions. In these models ornaments and preferences are determined by many genes (Mead and Arnold 2004). In the simplest of these models the genetic values are described by three parameters: the genetic variance for the ornament (G), the genetic variance for the preference (H) and the genetic covariance between the ornament and preference (B). Lande (1981) determined that the key parameter in determining whether evolution led to a stable or unstable equilibrium was the slope of the regression of B against G (figure 1.2b), therefore emphasising the importance of the covariance between trait and preference. Quantitative models incorporating indicator mechanisms, either through "good-genes" or "good-parents" (direct benefits), have also been formulated (Iwasa and Pomiankowski 1991; Iwasa and Pomiankowski 1999; Houle and Kondrashov 2002). "Good-genes" models largely rely on deleterious mutations to constantly reduce viability below its maximum (Iwasa and Pomiankowski 1991; Andersson 1994; Rowe and Houle 1996; Houle and Kondrashov 2002).

An interesting feature of several quantitative genetic models is the production of stable limit cycles of ornament evolution (Iwasa and Pomiankowski 1995; Mead and Arnold 2004). These differ from runaway processes in that the ornament and preference do evolve to an equilibrium but then progress along an elliptical path with periods of ornament elaboration and diminution (figure 1.2c). These have been suggested to be stronger under a pure Fisherian process (Iwasa and Pomiankowski 1999), although a "good-genes" model in which a Fisherian process was eliminated also found strong cycles (Houle and Kondrashov 2002). Quantitative genetic models have also been extended further to investigate the evolution of multiple traits. These appear to evolve most easily under a Fisherian process and can also undergo cyclical

evolution (Pomiankowski and Iwasa 1993; Iwasa and Pomiankowski 1994; Pomiankowski and Iwasa 1998). However a recent model incorporating a sexual conflict process also predicted the evolution of multiple ornaments and cyclical evolution (Moore and Pizzari 2005).

These models are useful in predicting the types of evolutionary scenarios that can occur under sexual selection. However the similarity of the outcomes under different models makes them difficult to discern without explicit tests of the underlying assumptions and parameters.

**Quantitative genetic studies of sexually selected traits in birds**

Studies of the quantitative genetics of sexually selected traits allow direct tests of some of the assumptions of quantitative genetic models of sexual selection. Early hybridisation studies have provided some information on the genetic basis of sexually selected traits. For example, hybridisation of Golden pheasants (*Chrysolophus pictus*) and Lady Amherst's pheasants (*C. amherstiae*) revealed that the divergent male plumage traits of these species were controlled by several loci (Danforth 1950). This suggests that the assumption of multiple loci in quantitative genetic models is realistic.

One of the key assumptions of "good-genes" models of sexual selection is that there must be heritable genetic variation in sexually selected characters. Artificial selection for increased comb size in the domestic chicken produced a response, demonstrating the presence of additive genetic variation (Von Schantz et al. 1995). A quantitative genetic study of the black breast-stripe in the great tit, a plumage colour trait used in mate choice, revealed significant heritable variation (Norris 1993). However a study on the black bib of male house sparrows found that this trait was entirely determined by environmental variation and lacked any additive genetic

component (Griffith et al. 1999a). The white male forehead patch in collared flycatchers has been the subject of several quantitative genetic studies. In a Swedish population, where forehead patch size is a condition-dependent trait used in mate choice (Gustafsson et al. 1995), there was significant heritability of this trait but only during years with favourable conditions (Qvarnström 1999a). During periods of unfavourable conditions there was no significant heritability of this trait and heritability also decreased with increasing maternal age. In a different collared flycatcher population in Hungary the size of the white wing patch, not the forehead patch, was condition-dependent (Török et al. 2003). In this population forehead patch size had slightly higher heritability than wing patch size and a corresponding higher heritability than forehead patch in the Swedish population. These studies highlight the complexity of genotype-by-environment interactions that can be found in condition-dependent traits. Although these interactions are likely to weaken any evolutionary response to sexual selection, they may also help to maintain genetic variation in the population by causing different genotypes to be selected under different conditions (Qvarnström 1999a).

A quantitative genetic study of a Swedish population of collared flycatchers was used to estimate the additive genetic variance of the male ornament (forehead patch size), the female choice for the ornament and male and female fitness (Qvarnström et al. 2006). The genetic covariances between these could then be estimated to determine the potential for "Fisherian" and "good-genes" sexual selection. There was some potential for a "good-genes" process because of a weak but positive genetic correlation between the ornament and male fitness. However there seemed to be very little potential for a Fisherian process as there was almost no correlation between mate choice and the male ornament. This was due to very low

additive genetic variance in female mate choice. A similar study on the blue cap colour of male blue tits, estimated the heritability of cap colour and fitness components in order to estimate the genetic covariance of these traits (Hadfield et al. 2006). This revealed very low heritability for both of these with large environmental effects on offspring fitness. Therefore, unsurprisingly there was also no genetic covariance between colour and offspring fitness, suggesting that a "good-genes" process is not at work in this population.

The zebra finch (*Taeniopygia guttata*) was used to test one of the assumptions of the immunocompetence-handicap hypothesis, namely that there is positive additive genetic covariance between body condition and immune response (Gleeson et al. 2005). This was found to be the case with over half of the genetic variation in body condition explained by genetic variation in immune response, at least in captivity. Another study in zebra finches illustrated the potential for intra-locus sexual conflict, in which the expression of a particular trait enhances the fitness in one sex but reduces it in another (Price 1996). Red bill colour appears to be a preferred condition-dependent trait in males but decreases fitness in females. Significant heritability was found in bill colour but also a strong genetic correlation between male and female bill colour, suggesting that there will be limited potential for the evolution of separate bill colour optima in the sexes.

**The molecular evolution of sexually selected traits**

Quantitative genetic studies are of most utility in identifying the potential for on-going sexual selection in existing populations. As can be seen from the above examples, they can produce very different results in different populations and under different conditions, meaning that the strength of sexual selection acting on a population can easily be over- or underestimated. In addition they assume that the

observed traits are produced as a result of many loci of small effect, which may not necessarily be the case (Charmantier and Sheldon 2006). The attraction of investigating sexual selection at the molecular level is therefore three-fold: firstly, historical events of sexual selection will leave signatures at the molecular level, which can be detected by comparing genetic sequence data from two or more species (Yang and Bielawski 2000); secondly, environmental variation is necessarily removed so genetic variation and change can be identified unambiguously; thirdly, the number of loci involved in producing a sexually selected trait and the size of the effect of these loci can be identified.

One of the main subjects of studies of the molecular evolution of sexually selected traits to date, are seminal fluid proteins. These have been found to be rapidly evolving in insects (Swanson et al. 2001a; Kern et al. 2004; Andrés et al. 2006) and primates (Jensen-Seaman and Li 2003; Dorus et al. 2004; Clark and Swanson 2005). This appears to be due to sexual selection in at least one case in which the rate of protein evolution was related to the level of intrasexual competition (Dorus et al. 2004), although there is some evidence that these proteins could also be involved in immune defence (Lung et al. 2001). Rapid evolution has also been found in gamete recognition proteins in a wide range of animals (Torgerson et al. 2002; Geyer and Palumbi 2003; Glassey and Civetta 2004; McCartney and Lessios 2004). In terms of sexual selection, the evolutionary dynamics of these proteins seem mostly likely to be the result of sexual conflict (Chapman et al. 1995; Andrés and Arnqvist 2001) and therefore may be different to the dynamics of premating sexual selection. However, a particularly interesting feature of these studies is the identification of rapidly evolving female proteins that may be involved in female-male interactions (Swanson et al.

2001b; Swanson et al. 2004) and so act as the female choice or resistance components of models of sexual selection or sexual conflict.

Significant progress has recently been made towards identifying the molecular genetic basis of a variety traits involved in mate choice in several species (Kopp and True 2002; Llopart et al. 2002; Sætre et al. 2003; Zauner et al. 2003; Kavaliers et al. 2005; Kronforst et al. 2006; Yeh et al. 2006). Many of these have involved linkage studies and have revealed some interesting features, such as the linkage between wing colour and female preference loci in *Heliconius* butterfly species (Kronforst et al. 2006). Similarly, male species-specific plumage characters in hybridising populations of pied and collared flycatchers (*Ficedula hypoleuca* and *F. albicollis*) were found to be Z chromosome linked, as were loci determining hybrid sterility, suggesting a role in speciation (Sætre et al. 2003). Once the molecular genetic basis of traits such as these has been identified we can begin to look at their evolution across species for evidence of sexual selection.

The sex chromosomes have been suggested to play an important role in the evolution of sexually selected traits (Reinhold 1998). This could be due to sexual antagonism, where an allele is beneficial to one sex but detrimental to the other. In female-heterogametic (Z-W) systems, such as birds, females would be expected in prefer males carrying Z-linked alleles beneficial to sons, as they will gain the fitness benefit of sexy sons and daughters will only inherit one of their father's Z chromosomes (Albert and Otto 2005). In contrast in male-heterogametic (X-Y) systems, such as mammals, females would be expected to prefer males carrying X-linked alleles beneficial to daughters. In many taxa, rates of mutation seem to be higher in males than females, perhaps due to the higher number of cell divisions per generation in the male compared to female germ line (Li et al. 2002; Makova and Li

2002; Bartosch-Härlid et al. 2003; Makova et al. 2004). This means that chromosomes that spend more time in the male than female line (Y or Z chromosomes) will tend to have higher mutation rates than both the autosomes and the other (X or W) sex chromosome (Axelsson et al. 2005). This could affect the rate of adaptive evolution of loci on the sex chromosomes. It has therefore been suggested that animals with Z-W sex determination should be prone to rapidly evolving male sexual displays and that these should be determined by Z-linked loci (Kirkpatrick and Hall 2004).

Both of the above hypotheses predict that the prevalence of sexually selected traits and their frequency of sex-linkage should vary between taxa with X-Y and Z-W sex determination systems. However this does not appear to be supported by the majority of empirical studies. For example, a comparative study of ray-finned fishes, in which both male-heterogamety and female-heterogamety are present, found no significant difference in the prevalence of sexually selected traits between these groups (Mank et al. 2006). There is some evidence that in birds, sexually selected traits may be biased towards the Z-chromosome (Sætre et al. 2003) but a survey of sexually selected traits in insects and mammals suggested that these also may be biased towards the X chromosome (Reinhold 1998). In contrast to this, however, a wide screen of male sex-biased gene expression in *Drosophila* found an under-representation of X chromosome genes and evidence that the X chromosome is an unfavoured location for male-expressed genes (Parisi et al. 2003); although in this study most of the male-biased genes were expressed in the germ line and therefore not involved in secondary sexual trait production. Overall it does appear that the sex chromosomes may be particularly important with regard to the genetics of sexually selected traits. However, the prevalence of sex-linkage among secondary sexual trait loci, the reasons for this, and its importance are still the subjects of much debate.

Further investigation into the genetic basis of a wider range of sexually selected traits across animal taxa is required to answer these questions.

## The genetic and developmental basis of feather ornaments

### Building a feather

Feathers are complex integumentary structures with hierarchical branching patterns (figure 1.3a). They are thought to have evolved from the scutate scales of the archosauran ancestor of birds and this evolution occurred independently from the origin of mammalian hairs (Harris et al. 2002). Feathers grow from the feather follicle (figure 1.3b). Here the epidermis becomes invaginated surrounding the feather filament, which is cylindrical in structure (Yu et al. 2004). At the centre of this cylinder is the feather pulp. This is the mesenchymal part of the feather and is involved in supplying nutrients to the developing feather but will not form a part of the final feather structure. The feather emerges as a conical structure surrounded by the feather sheath (figure 1.3c), which breaks away allowing the feather to unfurl. In typical bilaterally symmetrical feathers the barbs (first level of branches) are attached to a central rachis (figure 1.3a). This is achieved by helical organisation of the barbs such that new barbs are specified at the posterior side of the follicle and fuse to the rachis at the anterior side. In contrast in radially symmetrical downy feathers the barbs are specified vertically and only connected by the calamus at the base. Within the feather follicle, barbs are formed by keratinocytes, which are organised into the barb ridges. Between the barb ridges is the marginal plate epithelium, which eventually breaks down and does not form a part of the final feather. Within the barb ridges there is further organisation of the keratinocytes into two columns that form the proximal and distal barbule plates, which will eventually form the barbules (second level of branches). Between the barbule plates, the axial plate forms that will become the barb ramus.

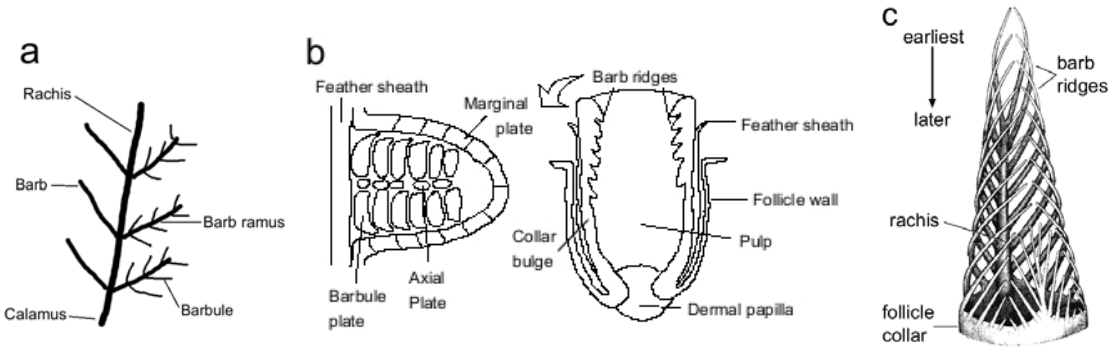



Figure1.3 Feather structure and development. **a**, the hierarchically branched structure of a typical feather. **b**, the structure of a developing feather follicle with an enlargement of a developing barb ridge to the left (adapted from Yu et al 2002). **c**, the conical structure of an emerging feather with helically oriented barbs (from Prum & Williamson, 2001).

Recent work has shed some light on the molecules involved in determining this structural complexity. The interaction of Sonic hedgehog (SHH), bone morphogenic proteins (BMP2 and BMP4) and noggin have been found to be involved in the formation of the early feather placode (undifferentiated feather bud) (Ting-Berreth and Chuong 1996; Harris et al. 2002) and then later in the formation of the barb ridges (Harris et al. 2002; Yu et al. 2002; Yu et al. 2004). *SHH* and *BMP2* expression patterns in the feather placode are similar to those seen in epithelial placode of avian tarsal scales and the placode of alligator scales, suggesting that at this stage the mechanisms of development have been evolutionarily conserved (Harris et al. 2002). Later in development *SHH* expression is limited to the marginal plate epithelium between the barb ridges (Ting-Berreth and Chuong 1996) and seems to be involved in specifying the fate of these cells to undergo cell death. Agonistic and antagonistic interactions between this and *BMP2*, which is coexpressed in the marginal plate but limited to the peripheral region, have been suggested to be sufficient to explain barb formation (Harris et al. 2002; Harris et al. 2005). *BMP2* and *BMP4* also seem to be expressed and involved in the differentiation of the barbule plates (Yu et al. 2002).

The helical nature of barb ridge propagation, however, requires the presence of an additional signalling molecule that is present in an anterior-posterior gradient

(Harris et al. 2005). Recent work suggests that this molecule may be *wingless int 3a (WNT3)* as this is present as an anterior-posterior gradient and perturbation can cause displaced or ectopic rachis formation (Yue et al. 2006). Work on stem cells in feather follicles determined that these are located in a region called the collar bulge (Yue et al. 2005) (figure 1.3b). The positioning of this ring of cells seems to be involved in determining feather symmetry; in bilaterally symmetrical feathers this was found to be angled in the anterior-posterior direction, whereas in radially symmetrical feathers it was not. This angle seems to be determined by cells in the dermal papilla of the feather follicle, suggesting that these may be responsible for setting up the *WNT3* gradient.

These findings point to a series of events leading to the evolution of modern bilaterally symmetrical avian feathers (Prum 1999). First, redeployment of the expression of *SHH* and *BMP2* could lead to the evolution of an elongate tubular structure from a primitive scale (Harris et al. 2002). Further modification of this signalling system into longitudinal strips of *SHH* and *BMP2* expression could then have resulted in a subdivision of the feather into several filamentous barbs. From this radially symmetrical feather a bilaterally symmetrical feather then evolved by *WNT3* expression determining anterior-posterior asymmetry (Yue et al. 2005; Yue et al. 2006).

Helical growth of feather barbs also has interesting implications for the formation of feather shape and patterning. This is because all parts of the feather grow at the same rate, yet the angle of the barbs relative to the rachis means that an isochronic section of cells in the feather follicle will not correspond to a straight line in the fully-developed feather. Mathematical models of feather growth were able to predict a range of observed feather shapes by altering the growth rate, angle of helical

growth, the number of barb ridges, their diameter and how fast new barbs were added (Prum and Williamson 2001). Similarly a model for within-feather patterning involving the same model of feather development and a simple reaction-diffusion model of pigmentation involving activation and inhibition signals was able to simulate a wide range of feather patterns that are observed in nature (Prum and Williamson 2002). The molecular determinants of these growth parameters, however, have yet to be determined.

**Melanin Synthesis and Deposition**

Melanins are the most widely used pigments in avian feathers. Most of the current understanding of how these pigments are synthesised and deposited in the integument comes from work done on mammals (Jackson 1997; Sturm et al. 2001). The work on birds (primarily quails and chickens) suggests that the process is very similar (Takeuchi et al. 1998; Wakamatsu et al. 1998; Tobita-Teramoto et al. 2000; Chang et al. 2006). In both mammalian hairs and avian feathers, two forms of melanin occur: eumelanin, which is dark brown to black in colour and pheomelanin, which is yellow to rufous brown in colour (Ito and Wakamatsu 2003).

Melanins are synthesised endogenously by specialised cells, the melanocytes. In birds, as in all vertebrates, these pigment-producing cells are derived from embryonic neural crest cells that migrate along the lateral pathway (Wakamatsu et al. 1998). In mammals, the *endothelin-3* (*Edn3*) and the *endothelin receptor-B* (*EDNRB*) genes are necessary for normal melanoblast (undifferentiated melanocyte) formation and migration from the neural crest (Jackson 1997). In birds it appears, from work done on a quail pigmentation mutation, that *endothelin receptor-B subtype-2* (*EDNRB2)* may have a similar role (Miwa et al. 2006). One of the primary factors in determining melanocyte differentiation is the *microphthalmia* gene (*MITF*) (Vance

and Goding 2004) and the activity of this in avian pigmentation has been confirmed via another quail pigmentation variant (Mochii et al. 1998). *MITF* encodes a basic helix-loop-helix transcription factor, which is essential for melanoblast survival (Carreira et al. 2005) and the transcription of melanocyte-specific proteins such as the tyrosinase enzymes (tyrosinase, TYR, and the tyrosinase related proteins, TYRP). BMP2 has also been shown to increase melanin synthesis via increased *TYR* transcription in birds (Bilodeau et al. 2001). This is interesting given that BMP2 is found in the same region of the feather follicle as the melanocytes during feather genesis. In the adult bird, melanocytes in the dermis migrate from the pulp of the feather follicle into the epidermis, where they form pseudopodia to transfer melanin into the keratinocytes (figure 1.4a).

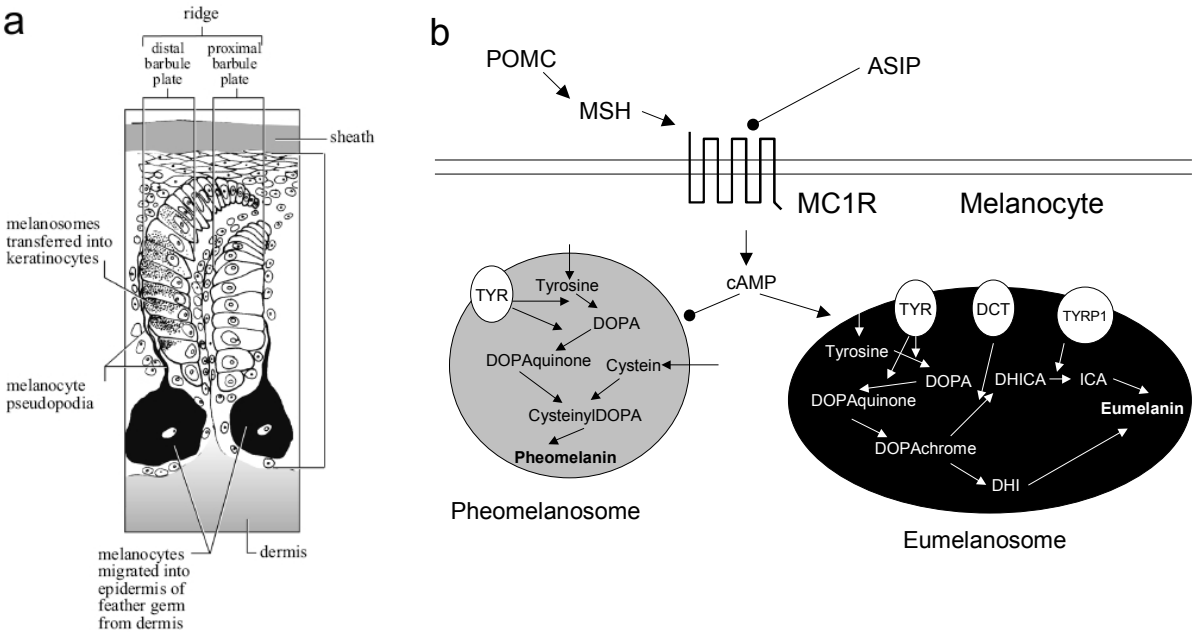



Figure1.4 Melanin synthesis and deposition. **a**, melanocytes within the feather follicle transferring melanosomes to the keratinocytes of the barb ridge (from Prum and Williamson, 2002). **b**, the developmental and biochemical pathways of melanin synthesis within the mammalian melanocyte. ➔ = pathway or positive induction, —• = negative induction.

Within the melanocyte, melanin synthesis occurs in lysosome-related organelles called melanosomes. Melanosomes synthesising pheomelanin

(pheomelanosomes) are distinct in structure from melanosomes synthesising eumelanin (eumelanosomes) (figure 1.4b) and it is thought that within a single melanocyte only one of these types of melanosome is produced. The production of eumelanosomes in both birds and mammals seems to be controlled by cell signalling events mediated by the melanocortin-1-receptor (MC1R) (Robbins et al. 1993; Takeuchi et al. 1998). This is a 7-transmembrane G-protein coupled receptor, situated in the melanocyte membrane. Mutations at *MC1R* are responsible for colour variants in a wide range of birds and mammals (figure 1.5). Activation of this receptor is via the melanocyte stimulating hormone (α-MSH) and leads to production of eumelanosomes via a signalling cascade causing elevation of intracellular cAMP (cyclic adenosine monophosphate) (Bilodeau et al. 2001). α-MSH is produced from the precursor molecule pro-opiomelanocortin (POMC) locally, within the skin (Gantz and Fong 2003; Boswell and Takeuchi 2005). In mammals there is also an endogenous antagonist of MC1R, the agouti signalling peptide (ASIP), which causes a switch to the production of pheomelanosomes (Siracusa 1994). Until recently *ASIP* was not know in birds (Klovins and Schiöth 2005) and it is widely thought that the agouti related protein (*AGRP*), which is expressed in avian skin (Takeuchi et al. 2000), may have a similar role in pigmentation in birds (Boswell and Takeuchi 2005).

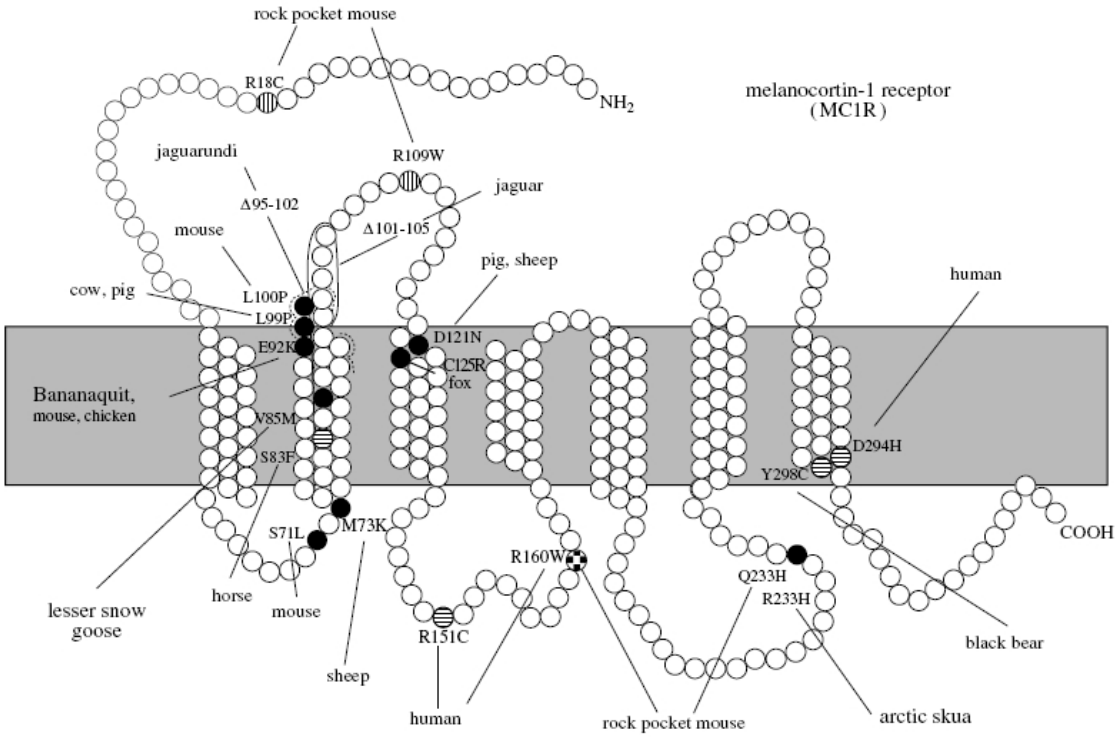



Figure1.5 MC1R variants associated with plumage or hair colour change. Extracellular is at the top. Amino acid substitutions are indicated using their single letter codes, Δ indicates deletions, numbers indicate positions in the species they refer to. Substitutions associated with increased eumelanin are shown in black and decreased eumelanin (and often increased pheomelanin) are shown with horizontal hatching. The R160W substitution is associated with eumelanism in rock pocket mice and red hair in humans. From Mundy (2005), for references see this and Majerus and Mundy 2003.

Eumelanosomes differ from pheomelanosomes in their membrane-bound enzymes as well as in several other structural components (Jimbow et al. 2000). Both types of melanosomes contain the rate-limiting enzyme, tyrosinase (TYR) (Ito et al. 2000). The activity of this enzyme in birds has been confirmed by the characterisation of two melanin-lacking mutants at this locus in chickens (Tobita-Teramoto et al. 2000; Chang et al. 2006). TYR catalyses the first two steps in the melanin synthesis pathway, which are from the amino acid tyrosine to dihydroxyphenylalanine (DOPA) and then to DOPAquinone. These steps are common to both eumelanogenesis and pheomelanogenesis. However beyond this the biochemical pathways differ (figure 1.4b). Tyrosine is therefore essential for both types of melanogenesis and seems to be

actively transported into the melanosome by an, as-yet uncharacterised, protein (Potterf et al. 1996). Eumelanosomes differ from pheomelanosomes in that they contain the tyrosinase-related proteins (tyrosinase-related protein 1, TYRP1 and DOPAchrome tautomerase, DCT), which catalyse the further steps required to produce eumelanin (Ito et al. 2000). These have been identified in chicken skin (April et al. 1998a; April et al. 1998b), although their activity in pigmentation in birds has yet to be demonstrated conclusively. Eumelanosomes are also unique in containing the PMEL17 protein, which is a membrane protein and a component of the fibrous striations upon which melanins are polymerised and mutations at this locus produce pigmentation defects in chickens (Kerje et al. 2004).

Other eumelanosomal proteins have been identified in mammals although their exact roles are less well characterised. One of these is the p-protein, which is responsible for human oculocutaneous albinism type 2 (OCA2) and may be involved in regulating melanosomal pH (Sturm et al. 2001). In contrast, pheomelanosomes appear to contain far fewer unique proteins and it has been proposed that they may not be "true" melanosomes (Jimbow et al. 2000). In the presence of cysteine no enzymatic processes are needed for the production of cysteinylDOPAs from DOPAquinone and from this to pheomelanin. However, high levels of the amino acid cysteine are needed (Ito et al. 2000). A gene affecting pheomelanin synthesis (*Slc7a11*) has recently been identified in mice and is proposed to code for a cysteine transport protein in the pheomelanosomal membrane (Chintala et al. 2005). This could have a crucial role in regulation of pheomelanogenesis and perhaps also in determining the shade of pheomelanin produced.

Once the melanosomes are formed and contain melanin they must be transported to the periphery of the melanocyte and transferred to a keratinocyte of the

developing feather. In mammals they are transported to the periphery by proteins in the kinesin superfamily along microtubule tracks. At the cell periphery the melanosomes are transferred to the actin cytoskeleton, which they attach to via a three-part complex made up of the proteins Rab27a, melanophilin (Mlph) and myosin Va (Barral and Seabra 2004). Mutations at the loci encoding at any one of these proteins cause abnormal pigmentation in mice. It appears that the lavender mutation in quails and chickens may also be due to a mutation at the avian *Mlph* locus (Vaez et al, unpublished), suggesting that this component, at least, is also required for melanosomal transport in birds.

**Carotenoids and other pigments**

Carotenoids are the second most common pigment type found in feathers and are red or yellow in colour. Unlike melanins they cannot be synthesised de novo by vertebrates and must be obtained in the diet. However, many birds convert dietary carotenoids, primarily lutein, zeaxanthin and β-carotene (which are yellow in colour), to different forms before depositing them in the feathers (Brush 1990). These can either be other yellow forms, such as the canary xanthophylls, or red forms such as astaxanthin and canthaxanthin. Both of these types of conversion require oxygenase enzymes, which have not yet been characterised, although mutant birds that apparently lack them have been described (McGraw et al. 2003c). The converted forms appear to be found in the blood plasma of birds suggesting that conversion takes place at a site prior to deposition (Hill 1995).

There are clearly other genetically controlled components required for carotenoid deposition in the feathers. Within the blood plasma, carotenoids are transported by lipoproteins (Deming and Erdman 1999; McGraw and Parker 2006), although the specificity of these is unknown. In some birds the type and amount of

carotenoids deposited in the feathers seem to be directly related to those present in the plasma (Hill et al. 1994; McGraw et al. 2001). However in other birds this is not the case (McGraw et al. 2003a; Tella et al. 2004), suggesting that there are specific mechanisms for accumulation and deposition of carotenoids in the keratinocytes of the feather follicle.

Carotenoid pigments have not been found in the plumage of any Galliformes or Anseriformes (McGraw 2006b), suggesting that these birds do not possess the necessary biochemical machinery for carotenoid deposition into the plumage although they are able to deposit carotenoids in the skin. This may suggest that the mechanisms for incorporating carotenoids in feathers evolved, perhaps from the mechanisms used to deposit them in the skin or bill, after the divergence of the Galliformes.

In addition to melanins and carotenoids some birds are able to utilise other pigment types in their feathers. These seem to be less widespread, but this may partly be due to the lack of widespread biochemical analysis of feather pigments and the general assumption is that most feather colours are due to melanins, carotenoids and structural colours. Other feather pigments include turacin and turacoverdin, which are red and green respectively and found in the turacos (Musophagidae). These copper-containing metalloporphyrins are synthesised endogenously (McGraw 2006a). Green pigments, that appear to be similar to turacoverdin, have also been described in several other bird species including two Galliformes, the blood pheasant (*Ithaginis cruentus*) and the roulroul (*Rollulus rouloul*), especially the female of this species (Dyck 1992). It has also been suggested that a unique form of pigment is involved in giving domestic chick feathers their yellow appearance and that this pigment is also present in penguin feathers (McGraw et al. 2004).

**The production of structural colours**

Structural colours are produced by nanostructural variations within the feather keratin that lead to differential scattering of light. Structural colours can be responsible for a wide variety of hues and can also be combined with pigments to produce more saturated hues than would be produced by the pigments themselves (Shawkey and Hill 2005). There are three types of structure that can produce colours within feathers. The first is unspecialised white feather keratin, which produces white by scattering all visible wavelengths of light. The second are found within the feather barbules and are composed of arrays of eumelanosomes connected by feather keratin (Zi 2003). These primarily produce iridescent colours. The third type involve the feather barb rami (main shaft of the barbs) and are composed of the spongy medullary keratin in which the feather keratin is interspersed with air pockets that act as light scatterers (Prum et al. 1999). In all galliform feathers studied to date the structural colours have been produced by melanin granules within the barbules (Prum 2006), illustrating the importance of melanin in the plumage displays of these birds.

Nothing is known about the genetics controlling the production of these colours beyond the normal processes of keratin and melanin synthesis outlined above. Developmentally it has been proposed that the biochemical structures are largely "self-assembling", determined by molecular interactions (Prum 2006), although this may be less true for melanosome-containing structures.

## Conclusions

The theoretical basis of sexual selection is a rapidly evolving field and to some extent empirical studies have failed to keep pace in testing the predictions of these models (Mead and Arnold 2004). As most models of sexual selection are formulated at the genetic level, molecular genetic studies may provide the clearest, least obscured empirical data against which these models can be compared.

Although feathers are complex structures that can be coloured by a variety of pigments as well as by structural scattering of light (Hill and McGraw 2006), the main pigments used are melanins. These pigments are also used to colour mammalian hairs and have been widely studied in this class (Jackson 1997). Studies on domestic fowl have identified several mammalian melanin synthesis genes in birds (Mochii et al. 1992; Takeuchi et al. 1996; April et al. 1998a; April et al. 1998b) and the sequencing of the chicken genome is bound to help in this regard (Consortium 2004; Klovins and Schiöth 2005). The role of these genes in pigmentation, however, has only been demonstrated in a handful of cases (Mochii et al. 1998; Takeuchi et al. 1998; Tobita-Teramoto et al. 2000; Kerje et al. 2004). Therefore plenty of scope exists for improving our understanding of avian pigmentation genetics by the investigation of candidate genes. In spite of this the potential for at least one of these genes (*MC1R*) to cause adaptive variation in the pigmentation of wild birds has already been demonstrated (Theron et al. 2001; Mundy et al. 2004; Mundy 2005). It therefore now seems feasible to use a candidate gene approach to investigate whether any of these genes are evolving in response to sexual selection.

## Thesis outline

- Chapter 2: I study the phylogenetic relationships within the Galliformes and construct a phylogenetic tree, which is then used to investigate the evolution of sexually dimorphic traits within these birds.
- Chapter 3: This phylogeny is then used to investigate evolution of several pigmentation genes and to look for signatures of sexual selection at the molecular level.
- Chapter 4: One of these genes, *MC1R*, is studied in more detail at the population level in the black grouse and red grouse to look for evidence of sexual selection at this level.
- Chapter 5: I investigate the role of a set of candidate genes in the production of colour variants of the Japanese quail and sexually dichromatic plumage colour in the common pheasant.
- Chapter 6: I discuss the implications of my findings and interesting avenues for future research.

# Chapter 2

# A phylogeny and comparative study of sexually dimorphic traits in the galliform birds


## Abstract

**In order to carry out comparative studies of any organisms, accurate phylogenies are needed. The galliform birds are an interesting group because of the wide range of sexual dimorphism that they exhibit and because they also include several well studied species. However, a well-resolved phylogeny has yet to be produced for this group. Here I use several methods to carry out a phylogenetic analysis of the Galliformes using a combination of mitochondrial, nuclear non-coding and nuclear coding loci. A combined Bayesian analysis of all of these loci produced the best-resolved phylogeny. This phylogeny was then used to investigate the evolution of three traits that may be evolving in response to sexual selection: male plumage colour, sexual size dimorphism and spurs. I found a correlation between sexual plumage dichromatism and sexual size dimorphism, and between relative male spur length and sexual dichromatism.**

## Introduction

The galliform birds offer a unique opportunity to study the evolutionary genetics of sexually selected plumage traits. This group contains several well-studied domestic species including the chicken, the only bird so far to have had its complete genome sequenced, and some of the best known and studied examples of sexual selection, such as the peafowl (Petrie et al. 1991). Additionally it contains both sexually dimorphic and monomorphic species making it an interesting group for studying the evolution of sexual dimorphism using comparative methods. In order to carry out comparative studies a well resolved and accurate phylogeny is needed (Harvey and Pagel 1990).

The Galliformes and the Anseriformes (waterfowl) are sister taxa and are the most basal orders of neognath birds (Fain and Houde 2004), thought to have originated in the Cretaceous (Tuinen and Dyke 2004; Pereira and Baker 2006). The family-level phylogenetic relationships within the Galliformes are relatively well established. The megapodes (Megapodiidae) are thought to have diverged first, followed by the curassows (Cracidae) (Pereira and Baker 2006). The New World quails (Odontophoridae) and guineafowl (Numididae) split off later, although the order of this is uncertain (Armstrong et al. 2001). All recent phylogenies suggest that the remaining taxa all belong to a single clade (Kimball et al. 1999; Dyke et al. 2003), which I will refer to as the Phasianidae, although it contains groups previously thought to be separate families such as the turkeys (Meleagridae) and grouse (Tetraonidae) (Dimcheff et al. 2002). The most complete molecular phylogeny of the Galliformes to date is that of Kimball et al (1999) (figure 2.1), which was based on mitochondrial cytochrome *b* sequence. This reveals a rapid radiation early in the evolution of the Phasianidae, which it fails to resolve. The use of only a single

mitochondrial locus is not ideal since this can lead to the gene tree not representing the true species tree (Hudson 1992). In order to overcome this it is necessary to sample several loci distributed throughout the genome. Some phylogenetic studies of the Galliformes have used intron sequence data from nuclear loci (Armstrong et al. 2001; Smith et al. 2005) but these have only sampled a narrow range of taxa.

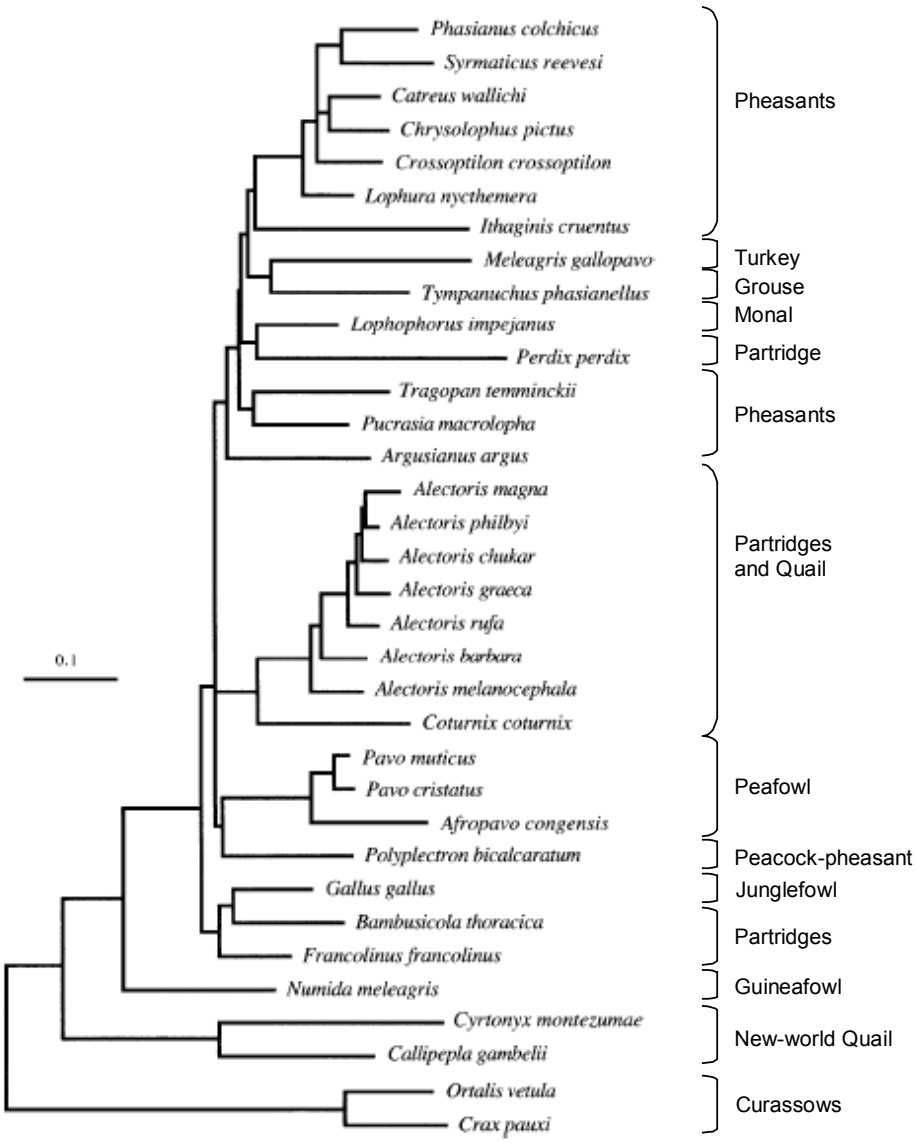



Figure 2.1 Kimball *et al.*'s (1999) phylogeny of the Galliformes (Fig 2): Most likely tree identified using cytochrome *b* nucleotide data.

My initial aim here is therefore to improve the resolution of the galliform phylogeny by using several nuclear and mitochondrial loci; primarily genomic non-coding DNA in the form of the introns of the agouti related protein gene (*AGRP*). This locus is located on the chicken chromosome 11 and is comprised of 2 introns of about 114bp and 981bp respectively. I also use the *CYTB* data from the Kimball study and extended the sampling of this locus to include additional taxa not included in that study. In addition, I use five nuclear coding loci: *AGRP*, the melanocortin 1-receptor gene (*MC1R),* the tyrosinase gene *(TYR),* the tyrosinase-related protein 1 gene *(TYRP1),* and the DOPAchrome tautomerase gene *(DCT)*. These are thought to be involved in feather pigmentation and were selected because they will later be used to investigate the molecular evolution of feather colour in the Galliformes but were included in this study simply to improve the power of the phylogenetic reconstructions. It is possible that these loci could bias the phylogeny as they may have evolved more rapidly on lineages with more divergent plumage colour. I therefore perform the phylogenetic reconstructions both with and without these loci in order to assess their impact on the phylogeny.

Once a well-resolved phylogeny has been obtained it can be used to investigate the evolution of traits that may be under sexual selection. Until recently the most widely used method for inferring the evolution of a trait over a phylogeny was maximum parsimony (Maddison and Maddison 2000). This was based on the intuitive assumption of minimising the amount of change in a trait over the phylogeny, meaning that the most closely related taxa will tend to be most similar (Harvey and Pagel 1990). This can work well for some traits. However, it makes no allowances for branch length, meaning that just as much change is assumed to have occurred on long branches as short. More importantly it will also encounter problems if traits change

rapidly and so may have undergone multiple changes along a single branch of a phylogeny (Webster and Purvis 2002). In order to overcome these problems, maximum-likelihood based methods have been developed (Pagel 1994; Pagel 1999b). These tend to be computationally intensive if a complete reconstruction of states at all ancestral nodes on a phylogeny is required. However, they can be very useful for testing hypotheses about the evolution of a particular trait or traits (Pagel 1999a). This can include features such as the timings of evolutionary events, the tempo of evolution and correlations between the evolution of different traits.

The main focus of this study will be plumage colour. Plumage colours in the Galliformes are primarily produced by melanins and fine feather structures, and carotenoids are not thought to be used (Smyth 1990; McGraw 2006b). In order to identify plumage colours that are used for sexual signalling by males, I will initially investigate sexual plumage dichromatism (the difference in plumage colour between males and females). Sexual dichromatism is widely accepted as a measure of pre-mating sexual selection (Sorci et al. 1998; McLain et al. 1999; Prinzing et al. 2002; Morrow and Pitcher 2003; Morrow et al. 2003) and several studies have found a correlation with other measures of sexual selection such as mating system (Andersson 1994; Figuerola and Green 2000; Dunn et al. 2001) or extra-pair paternity (Owens and Hartley 1998; Bennett and Owens 2002). However dichromatism could be associated with other factors such as differential habitat use by males and females or loss of showy plumage in females due to predation pressure (Irwin 1994; Burns 1998; Kimball and Ligon 1999). To overcome this problem I introduce a measure of male "showiness," which is similar to dichromatism but is a comparison of adult male colour to juvenile male instead of female colour. All of the species included in this study are ground-nesting, with the exception of the black curassow, so it seems

unlikely that females or juveniles should exhibit showy plumage traits. All juveniles appear brown and cryptic to the human observer and are generally sexually monomorphic. These measures have the advantage over "brightness" scores (Scott and Clutton-Brock 1990) in that they do not require judgments to be made about the relative brightness of different colours, which is dependent on the background against which they are normally viewed.

A great deal of emphasis has recently been placed on the importance of objective scoring of plumage colour (Cuthill et al. 1999; Eaton 2005). This is primarily because of the differences in the visual system of birds and humans. Birds have four spectrally distinct cone classes, compared to just three in humans. In some birds one of these is sensitive to ultra-violet (UV) (Ödeen and Hastad 2003) meaning that they can perceive colours outside the range of human vision. However, in all galliform species studied to date, the "violet" cone has a peak sensitivity similar to that of the "blue" cone of humans (Bowmaker et al. 1993; Bowmaker et al. 1997; Hart et al. 1999; Hart 2002). This means that differences in colour perception between Galliformes and humans may not be as pronounced or potentially important as in other bird species.

In addition to plumage colour I also investigate two other traits that may be the result of sexual selection: sexual size dimorphism (difference in body size between males and females) and male spurs. These are generally expected to more strongly be associated with the presence of male-male competition as compared to plumage dichromatism, which may be expected to be associated with female choice (Davidson 1985). Tarsal spurs are unique to the Galliformes and are composed of a bony core normally covered in a horny cap (Davidson 1985). These are generally larger or more numerous in males than females (Madge and McGowan 2002) and in some cases are

known to be used as weapons in male-male contests (Grahn et al. 1993). This has been assumed to be their main function (Davidson 1985), although there is some evidence that spurs are condition-dependent and may be used in female choice (Von Schantz et al. 1989; Møller 1992; Mateos and Carranza 1996). Very few comparative studies have considered spurs. One found a relationship between the presence of spurs and social mating system (scored simply as monogamous or polygamous) and sexual size dimorphism. However this did not take phylogenetic non-independence into account (Davidson 1985).

Sexual size dimorphism has been shown to increase with increased male-male competition in shorebirds (Lindenfors et al. 2003; Szekely et al. 2004) and to be associated with social mating system in these and other birds (Owens and Hartley 1998; Dunn et al. 2001), although not in the Anseriformes (Figuerola and Green 2000). In addition, in both of the studies in which there was a positive association, changes in size dimorphism were mostly due to changes in male not female body size. A further comparative study failed to find an association between lekking and size dimorphism in a range of bird taxa (Hoglund 1989) but did find an association when considering only the grouse.

Many of the Galliformes are sexually dimorphic in colour and size as well as possessing spurs (and often other ornaments as well that are not considered here such as wattles). It is not clear what role such "multiple ornaments" play. They may signal different aspects of male fitness signals or more simply act as back-ups (Johnstone 1996) or one or more of the traits may have lost its signalling component (Møller and Pomiankowski 1993; Ligon et al. 1998). If traits are correlated across lineages it may indicate that they are both functional. However, if they are not correlated, it may be

more likely that one has lost its function or equally that they are simply alternative functional signalling strategies.

My aim here is to investigate the evolution of spurs and sexual dimorphism in plumage colour and size over the galliform phylogeny. This is firstly to assess their potential as indicators of the strength of sexual selection. Perhaps the best and mostly widely accepted way of doing this would be to investigate the evolution of these traits in relation to social mating system, as this may give a measure of the theoretical potential for sexual selection. However, this is not currently possible for these birds given the lack of reliable information for many of them regarding their mating system in the wild. I also aim to identify the most appropriate methods for reconstructing the evolution of these traits. This is with a view to using one or more of these traits as a measure of the strength of sexual selection in subsequent studies into the evolution of plumage colour genes in response to sexual selection in these birds.

# Methods

## Laboratory Methods

Genomic DNA was extracted from samples of soft tissue, blood or feather from 36 galliform species in 25 genera (see appendix 2 for sample list), using standard methods. I sequenced *AGRP* introns 1 and 2. *CYTB* sequences were downloaded from GenBank or sequenced as described previously (Kimball et al. 1999). I also sequenced 859bp of the *MC1R* exon, 772bp of *TYR* exon 1, 267bp of *TYRP1* exon 1, 233bp of *DCT* exon 2 and 432bp of the *AGRP* coding sequence (comprising exon 2 and parts of exons 1 and 3) (see table 2.1 for primer sequences). PCR reactions were performed in a 50µl total reaction containing: 1.0 units Taq polymerase (Advanced Biotechnologies), 1 x Reaction buffer, 1.5mM $MgCl_2$, 50mM each dNTP, 10nM each primer and 50-200ng DNA. PCR reactions were performed in a DNA Engine (MJ Research) with the following cycling parameters: 94ºC for 2 minutes, 35 x: (94ºC for 30s, 55-65ºC for 45s, 72ºC for 1 minute), 72ºC for 5 minutes. PCR products were directly sequenced on both strands by cycle sequencing using Big Dye v.3.1 (PE Biosystems) under standard conditions. Table 2.1 shows primers used for PCR and sequencing. Sequences were edited in Seqman 5.05 (DNASTAR Inc.). Sequences were aligned using clustal W and adjusted manually (see appendices 4-9 for sequences and alignments).

| Primer name | Sequence (5'-3') | Locus | Direction | Position |
|---|---|---|---|---|
| AGRPF1 | CCCAGGACCATGCTGAAC | *AGRP* | Forward | 25 |
| AGRPF4 | ATGCTGAACGTGCTGCTG | *AGRP* | Forward | 34 |
| AGRPF17 | CCAGCTCCCCAGCACTAAG | *AGRP* | Forward | 244 |
| AGRPF11 | ACCTTGCCCAGCCTCTCT | *AGRP* | Forward | 294 |
| AGRPF15 | GCCAAGAGCTGGTGTATTCC | *AGRP* | Forward | 427 |
| AGRPF3 | AGCCACGTCAGGCTTCTCT | *AGRP* | Forward | 475 |
| AGRPF8 | ACGGAGCAAACACTGAACG | *AGRP* | Forward | 510 |
| AGRPF9 | CTGCAGAGGACCAGACTTCC | *AGRP* | Forward | 952 |
| AGRPF7 | GGAGCTCTTCGCAGACTTTG | *AGRP* | Forward | 1169 |
| AGRPF2 | CAAAGCATGAAGAGGAGCTG | *AGRP* | Forward | 1212 |

| Primer name | Sequence (5'-3') | Locus | Direction | Position |
|---|---|---|---|---|
| AGRPF14 | CTCAGGCTCTGGGCTCTCTA | *AGRP* | Forward | 1318 |
| AGRPF16 | GTGGACCATGAGCCTCCTCT | *AGRP* | Forward | 1360 |
| AGRPF10 | CCTGCAGGCACTGTCCAC | *AGRP* | Forward | 1392 |
| AGRPR19 | GGGACTCACCATCAAGCTCT | *AGRP* | Reverse | 217 |
| AGRPR12 | GCTTCAGTTTTGGGGACCT | *AGRP* | Reverse | 264 |
| AGRPR13 | AAACTCTCAGTGTGTTACCTGTGG | *AGRP* | Reverse | 435 |
| AGRPR10 | ACAGAGGAGCCTGATGTGG | *AGRP* | Reverse | 495 |
| AGRPR2 | GGGCACAGAGAAGCCTGA | *AGRP* | Reverse | 499 |
| AGRPR5 | CCGTGAAGTGTTTGCTCCA | *AGRP* | Reverse | 528 |
| AGRPR7 | GCAGCGTTCAGTGTTTGCT | *AGRP* | Reverse | 536 |
| AGRPR9 | ATGGAAGTCTGGTCCTCTGC | *AGRP* | Reverse | 973 |
| AGRPR14 | AGGAAACAACCCTGGTCTCTC | *AGRP* | Reverse | 1082 |
| AGRPR16 | GGATTCTCCTCTTCCCATCC | *AGRP* | Reverse | 1165 |
| AGRPR8 | CGAAGAGCTCCTTGGGAAT | *AGRP* | Reverse | 1179 |
| AGRPR3 | GGCATCATCTGTGGAGACCT | *AGRP* | Reverse | 1190 |
| AGRPR17 | TGAGCACAATGGACCTATGG | *AGRP* | Reverse | 1208 |
| AGRPR11 | GAGGCACCTCCATCTCAAAC | *AGRP* | Reverse | 1321 |
| AGRPR18 | TTAGAGAGCCCAGAGCCTGA | *AGRP* | Reverse | 1339 |
| AGRPR6 | AGTAGCAGAAGGCGTTGAAGA | *AGRP* | Reverse | 1552 |
| AGRPR4 | AGGTGGTGCTGATCTTCCTG | *AGRP* | Reverse | 1573 |
| AGRPR1 | CCACATGGGAAGGTGGTG | *AGRP* | Reverse | 1583 |
| CYTBF1 | CGCTCAATCCCAAACAAACT | *CYTB* | Forward | 847 |
| DCTF2 | TGCTGGCTATAACTGTGGTGA | *DCT* | Forward | 309 |
| DCTR1 | CTGACAGAGTAGTAATGAAGCCAAA | *DCT* | Reverse | 587 |
| MSHR72 | ATGCCAGTGAGGGCAACCA | *MC1R* | Forward | 44 |
| MSHR80 | GTGCCAGGGGTTGGACAT | *MC1R* | Forward | 104 |
| MSHR9 | CTGGCTCCGGAAGGCATAGAT | *MC1R* | Reverse | 900 |
| MSHR78 | CAGGAGCACAGCACCACCTC | *MC1R* | Reverse | 941 |
| TP1e1F3 | CTCAGTTCCCTCGCCAGT | *TYRP1* | Forward | 68 |
| TP1e1R1 | GATTTGCTGGCTACAGGTAGGTC | *TYRP1* | Reverse | 375 |
| TYR1F | CTGTTTGCCATGGGCTTACT | *TYR* | Forward | 7 |
| TYR3F | GGCTTACTGCTGGTCATCCT | *TYR* | Forward | 19 |
| TYR4F | CAGTTCCCCAGAGCCTGT | *TYR* | Forward | 55 |
| TYR5F | CACCGTCAGTGAGAAGGACA | *TYR* | Forward | 378 |
| TYR2F | GGACAAGTTCCTTGCCTACCT | *TYR* | Forward | 394 |
| TYR2R | TGCTAGGTTAAGGTAGGCAAGG | *TYR* | Reverse | 423 |
| TYR4R | TGTCTGCCACCCATGTATTC | *TYR* | Reverse | 767 |
| TYR3R | AAAATGATGCTGGGCTGAGT | *TYR* | Reverse | 805 |
| TYR1R | CTGCCATGAGGAGAAAAATGA | *TYR* | Reverse | 819 |

Table 2.1 Primers used for PCR and sequencing. Positions are of the 5' end of the primer, relative to the start codon on the chicken sequence. Initial primers were designed based on chicken sequences with accessions: AB029443, D78272, D88349, AF003631 and AF023471

**Phylogenetic Analysis**

*AGRP* intron and *CYTB* sequences were used separately to reconstruct trees using Neighbour-Joining and Maximum Likelihood with an HKY85 model of substitution

(as this allows for unequal base frequencies and a higher rate of transitions than transversions and shows a good fit to most nucleotide data, Page and Holmes 1998) in PAUP* Version 4b10. In addition a maximum-parsimony analysis of a combined data set of all sequence data was carried out. These trees were all broadly similar to each other and to those published previously, with no or very little improved resolution.

In order to improve the resolution, two combined (partitioned) data sets, one of *AGRP* introns and *CYTB*, and one containing these plus the coding sequences for the 5 nuclear genes, were analysed using MrBayes v3.0 (Huelsenback and Ronquist 2001). The coding sequences were also partitioned into their three constituent codon positions and priors set to allow the sites specific rates model to vary across partitions. The likelihood model was set to allow transitions and transversions to have potentially different rates (as in a K80 or HKY85 model). The program was run with 550,000 generations, discarding the first 50,000 as burn-in and subsequently sampling every 100 generations. Fifty-percent consensus trees for these data sets were similar although with improved resolution for the larger data set. These combined data sets were also analysed using maximum-parsimony with all characters equally weighted. Phylogenetic trees were rooted to the Cracidae, as this family is fairly well established to be basal within the Galliformes (Pereira and Baker 2006). The Megapodiidae were not included in the phylogeny because *AGRP* data were not obtained for any taxa in this family.

**Character State Scoring.**

Sexual plumage dichromatism (hereafter referred to as dichromatism) was scored from field guide information (Jones et al. 1995; Madge and McGowan 2002) and illustrations on a scale of 0 to 6. This was based on three body regions (head and neck; back, wings and tail; chest belly and legs). Each region was scored from 0 to 2,

where 0 was no difference in plumage colour between males and females, 1 was a difference of shade or feather patterning and 2 was a difference in colour. Scoring was performed by two independent observers and found to have high repeatability ($r = -0.89$, $p<0.001$, intraclass correlation coefficient). The mean of the two scores was used. This scoring system was based on that of Owens and Bennett (1994), which has been widely used and found to correlate with other measures of the strength of sexual selection.

A simplified dichromatism score on a three point scoring system of 0 (monochromatic), 1 (slight dichromatism – difference in shade or feather patterning) or 2 (dichromatism – difference in colour or overall pattern) was also obtained. To check that my measure of dichromatism was not strongly influenced by female showiness, "male showiness" scores were obtained using the same criteria as the dichromatism scores but by comparing adult male breeding plumage to juvenile plumage, rather than adult female plumage.

Sexual size dimorphism was scored as $\log_{10}$(mwt/fwt) (Owens and Bennett 1994), where mwt and fwt are the mean male and female body masses respectively (data from Owens, personal communication). The presence of male spurs was recorded and scored as a binary presence/absence character. Relative male spur length was also scored as maximum male spur length/ male wing length (as a measure of male body size), with data obtained from Davison (1985).

**Maximum-Parsimony Analysis of Trait Evolution**

Maximum-parsimony reconstructions of sexual dichromatism and male spurs were carried out with MacClade 4 using the Bayesian consensus tree and the maximum-parsimony consensus tree. The latter included several polytomies, which were set as 'soft' in MacClade, meaning that they represented uncertainties in the resolution of

the phylogeny, not multiple speciation events. Any additional species that were not included in the phylogenetic study, but seemed as though they may affect the parsimony reconstruction (i.e. differed in dichromatism from closely related taxa), were included in this analysis, with their phylogenetic positions inferred from previous studies (Bloomer and Crowe 1998; Kimball et al. 2001; Lucchini et al. 2001; Dimcheff et al. 2002).

**Maximum-Likelihood Analysis of Trait Evolution**

The software package 'Continuous' was used to investigate the evolution of sexual dichromatism in the Galliformes (Pagel 1994; Pagel 1999b). This package uses a generalised least squares (GLS) model of trait evolution, which allows inferences to be made regarding the evolution of a single trait and tests for correlations between different traits. I tested for a dominant direction of change by comparing model A (constant variance random walk) to model B (directional random walk). The influence of branch length on trait evolution was tested using the parameter $\kappa$, where $\kappa=1$ indicates gradual evolution, whereas $\kappa=0$ indicates punctuational evolution. The importance of total path length on trait evolution was tested using the parameter $\delta$, where a low $\delta$ indicates that temporally early change is relatively more important and a high $\delta$ indicates that temporally later change is more important. The influence of phylogeny on trait evolution was tested using the parameter $\lambda$, which reveals the impact of phylogeny of trait evolution. Each parameter was investigated separately: a model with the parameter of interest equal to one was compared to a model with the parameter estimated from the data. The log likelihood values from these models were then compared using likelihood ratio tests (LRTs) to reveal whether parameters differed significantly from the default expectation (df=1). Model A with all parameters set to one is equivalent to an independent contrasts analysis (Felsenstein

1985). Covariance between traits was tested with a LRT comparing a model with covariance equal to zero to a model with covariance allowed (df=1). Correlation values were calculated within 'Continuous', with significance values equal to those of the covariances (Pagel 1994).

## Results

### Phylogeny of the Galliformes

The phylogeny produced for the *AGRP* intron sequences was very similar to the *CYTB* phylogeny, with similar lack of support for the deeper branches, but some strong support for branches not previously identified (figure 2.2). The Bayesian consensus tree from the large data set had the best resolution and was therefore used as the main phylogeny for the comparative analyses. This tree was very similar to that from the smaller data set (i.e. excluding the nuclear coding regions), suggesting that these do not have a large influence on the phylogeny (figure 2.3). In addition many of the groupings identified by the Bayesian analysis were also supported by the maximum-parsimony analysis (appendix 3, bootstrap values given in figure 2.3) suggesting that they are robust to the different assumptions of these models.

The roulroul partridge (*Rollulus rouloul*) was found to be basal within the main Phasianidae clade in all analyses. As in the Kimball study (figure 2.1), the jungle fowl (*Gallus*) grouped with the francolins (*Francolinus* and *Scleroptila*) and the Old-world quail (*Coturnix*) with the *Alectoris* partridges. The two francolin species (*Francolinus pondicerianus* and *Scleroptila levaillantoides*) were not consistently grouped together. This may have been partly influenced by missing data for *S. levaillantoides* at *CYTB* (only partial sequence was available) as can be seen from the ambiguous placement of this species in the *CYTB* phylogeny (figure 2.2b), although placement of these species relative to *Gallus* was also poorly resolved when using the other locus (figure 2.2a). The monophyly of the francolins has previously been questioned (Bloomer and Crowe 1998) and these two species assigned to separate genera and I have therefore chosen to use this nomenclature. Furthermore, *F. pondicerianus* is an Asian francolin, while

*S. levaillantoides* is one of the African species, therefore, it does seem feasible that these taxa could be polyphyletic with respect to the jungle fowl.

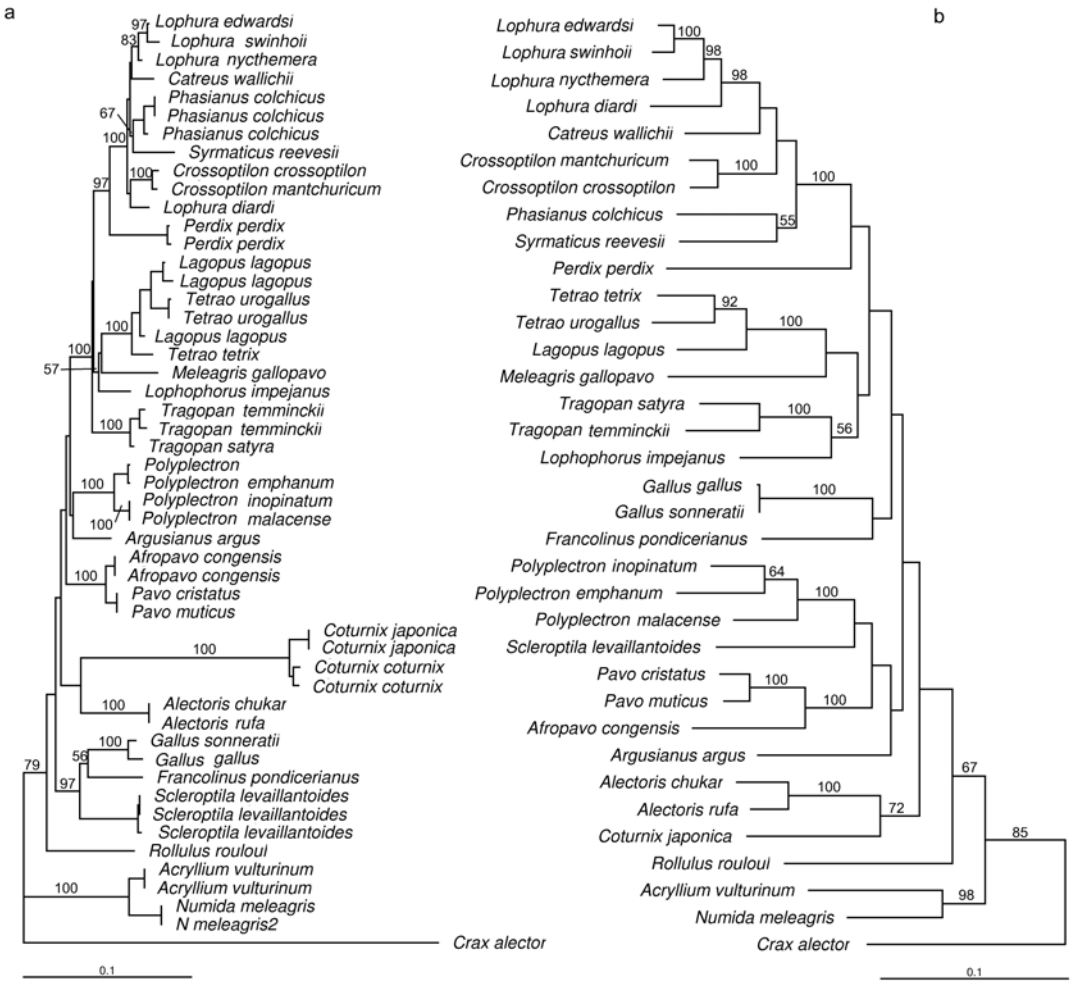



Figure 2.2 Maximum-likelihood phylogenies based on *AGRP* introns (**a**) and *CYTB* (**b**) using the HKY85 model of substitution. Neighbour-joining bootstrap values with 1000 replicates are given above branches with >50% bootstrap support.

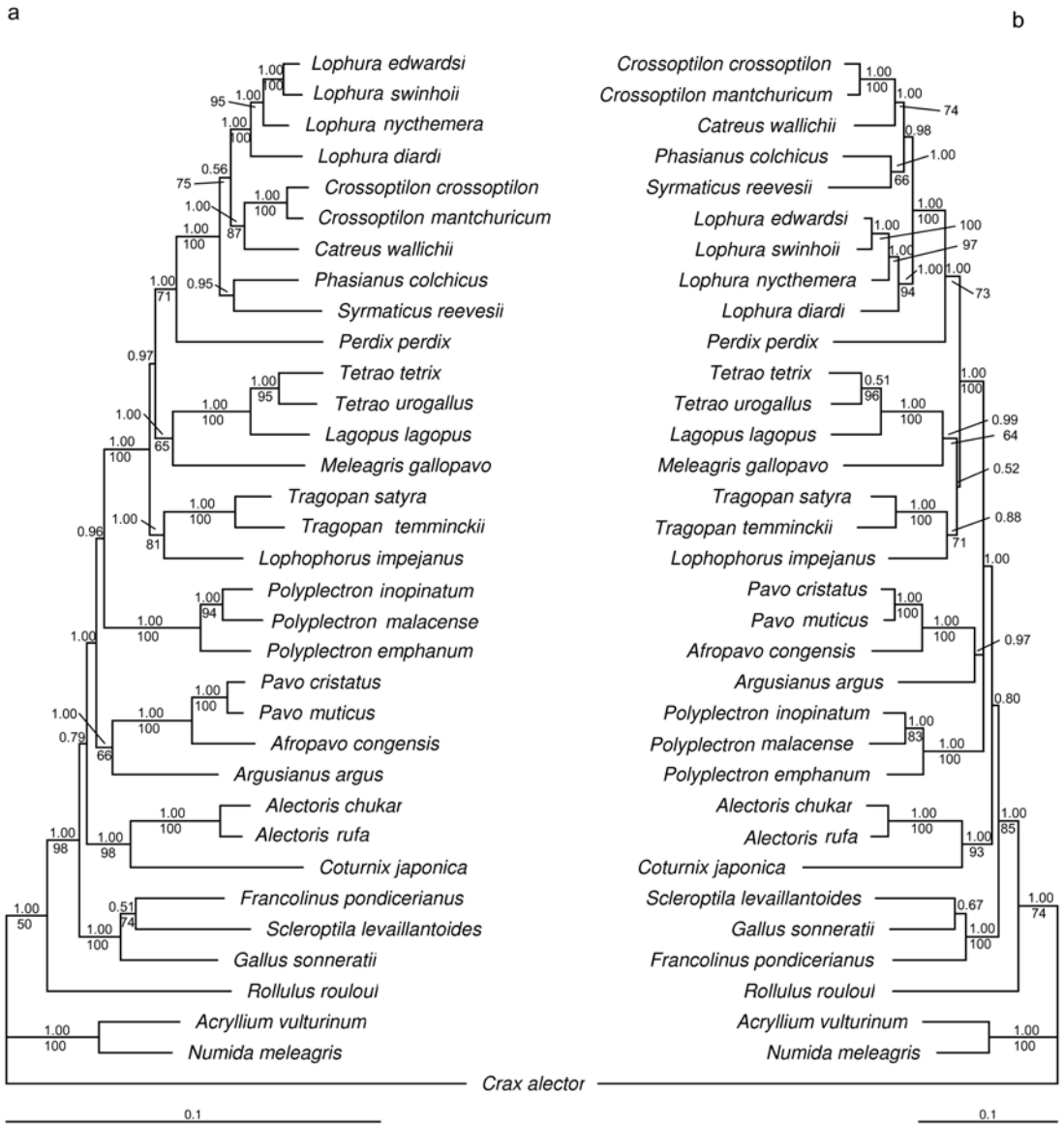



Figure 2.3 Bayesian MCMCMC phylogenies based on *AGRP* intron, *CYTB* and *MC1R, AGRP, TYR, TYRP1* and *DCT* sequence (**a**) and *AGRP* and *CYTB* only (**b**) with 550,000 generations, discarding the first 50,000 as burn-in and sampling every 100 generations. Bayesian probabilities are given above branches. Maximum-parsimony bootstrap values with 100 replicates for each of these data sets are given below branches with >50% bootstrap support.

I found very little support for the grouping of the peafowl (*Pavo/Afropavo*) and peacock pheasants (*Polyplectron*) that had been proposed previously. In agreement with Kimball et al. (1999) I found strong support for a group I will refer to as the

higher pheasants containing the eared-pheasants (*Crossoptilon*), the cheer pheasant (*Catreus*), the gallopheasants and firebacks (*Lophura*), the common pheasant (*Phasianus*) and the long-tailed pheasants (*Syrmaticus*). In addition I found strong support for a clade containing the higher pheasants, grey partridge (*Perdix perdix*), grouse (*Lagopus* and *Tetrao*), tragopans (*Tragopan*), monal (*Lophophorus impejanus*) and turkey (*Meleagris gallopavo*). Within this I found strong support for the grey partridge being a sister group to the higher pheasants. There was some support for the turkey being a sister group to the grouse. I also found evidence for a close relationship between the monal and the tragopan genera. The combined analysis supported a basal position for the junglefowl within the Phasianidae. It also revealed the argus pheasant to be sister to the peafowl and the cheer pheasant sister to the eared-pheasants.

**Maximum-Parsimony Analysis of Trait Evolution**

Reconstructions of sexual plumage dichromatism (scored from 0 to 6) over two different galliform phylogenies resulted in some ancestral nodes that could not be resolved unequivocally (figure 2.4), particularly if the phylogeny was not fully resolved (appendix 3). In order to resolve some of these nodes, the simplified dichromatism score (from 0 to 2) was used. This resulted in better resolution but at a cost of loss of information on the trait of interest (figure 2.5).

A comparison of the sexual dichromatism and male showiness reconstructions reveals that these are largely similar. This was expected given that the Galliformes are largely ground nesting birds and therefore female crypsis is likely to be important for camouflage during incubation. However there is a general trend for male showiness scores to be higher than dichromatism scores, indicating a tendency for females to be showier then juveniles. In only a few cases was this particularly evident, the eared pheasants (*Crossoptilon*), the rock partridges (*Alectoris*) and the guineafowl

(*Acryllium* and *Numida*). Information on the juvenile phenotype was not available for all the Cracidae. The ancestral state could not be reconstructed unambiguously.

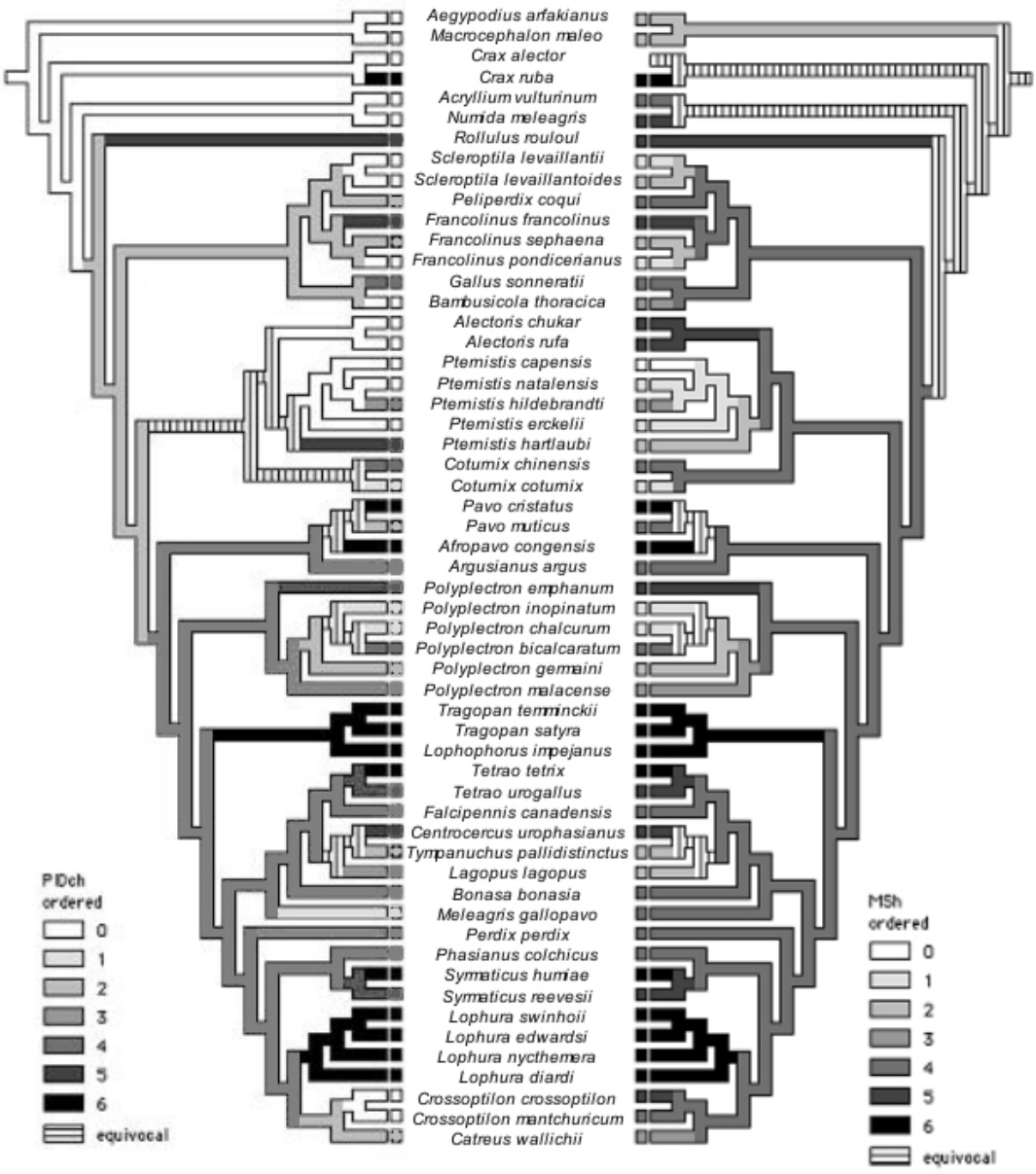



Figure 2.4 Reconstructions of sexual plumage dichromatism (left) and male showiness (right) evolution. Both were scored from 0-6 based on three body regions score from 0-2. Males were compared to females to obtain dichromatism scores and to juveniles to obtain showiness scores.

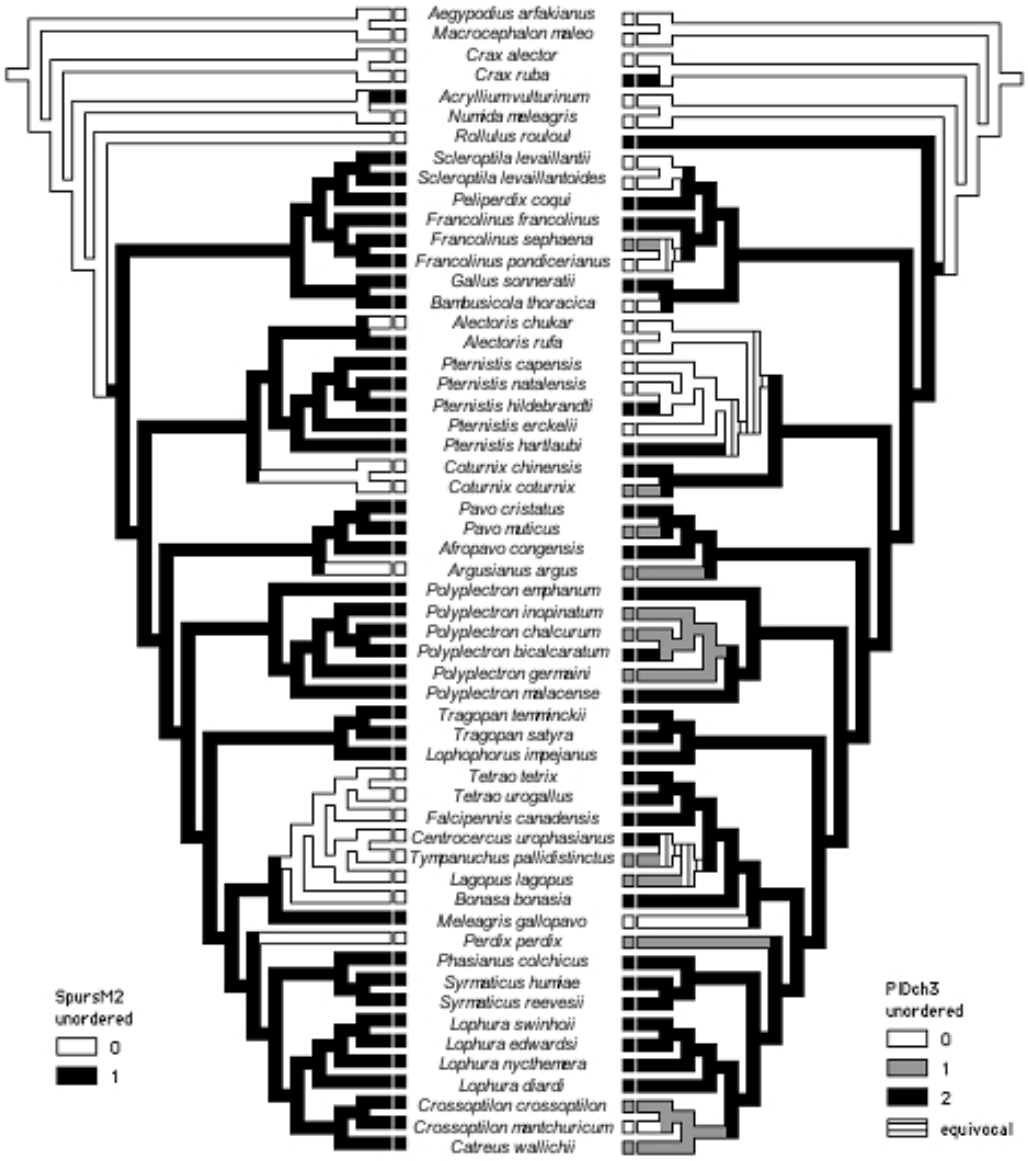



Figure 2.5 Reconstructions of spur evolution (left) and sexual plumage dichromatism scored from 0-2 (right).

Well-resolved reconstructions of spur evolution were obtained using this method (figure 2.5). These indicate an early origin of spurs in galliform evolution followed by multiple losses. The only exception to this is the vulturine guineafowl (*Acryllium vulturinum*), which appears to have evolved spurs independently. These are not full spurs but are described as "spur-like lumps" (Madge and McGowan

2002). This pattern of evolution is similar to that seen for the simplified scores of sexual dichromatism, as this also shows an early increase in dichromatism followed by several losses. However, there is very little correspondence between the lineages that have lost dichromatism and those that have lost spurs, suggesting that these two potential indicators of sexual selection are responding to different selection pressures.

**Maximum-Likelihood Analysis of Trait Evolution**

Model B was not significantly better at modelling evolution of any of these traits, suggesting that they do not show significant directional evolution. Sexual size dimorphism evolution showed a δ value significantly greater than one (δ=3, LRSt (likelihood ratio statistic) =8.31, p=0.004 – table 2.2) suggesting that temporally later change is relatively more important in its evolution. In addition λ was significantly less than one (λ=0.660, LRSt=3.85, p=0.050) and not significantly different from zero (LRSt=0.446, p=0.504) indicating that phylogeny has a minimal and non-significant effect on its evolution. Together these results indicate that size dimorphism has undergone rapid species-specific adaptation, with very low phylogenetic constraint.

| Parameter<br>Action | κ<br>Scale branch lengths | δ<br>Scale total path (root to tip) | λ<br>Contribution of phylogeny |
|---|---|---|---|
| Size Dimorph. | 0.426 | 3.00** | 0.660* |
| Dichromatism | 0.00** | 0.763 | 1.00 |
| Showiness | 0.423 | 1.45 | 0.859 |
| Spur presence | 1.14 | 0.977 | 0.952 |
| Spur length | 0.845 | 1.40 | 1.00 |

Table 2.2 Maximum-likelihood estimates of the 3 evolutionary parameters for each of the 4 traits studied: sexual size dimorphism, sexual dichromatism, male showiness and spurs. * indicates significance relative to the default value of 1; * $p<0.05$, ** $p<0.01$.

As expected dichromatism and male showiness scores were highly correlated ($r^2$=0.53, p<0.001 – table 2.3) and showed similar patterns of evolution. Both of these traits showed evidence of punctuational evolution, with κ values not significantly different to zero (LRSt=0.00, p=1.00 and LRSt=1.12, p=0.29 for dichromatism and showiness respectively). This would be consistent with these traits having a role in speciation through mate choice. In addition the values of λ and δ were not significantly different from one (LRSt=0. 00 p=1.00, and LRSt=0.63, p=0.426 respectively for dichromatism) indicating that there is a significant effect of phylogeny and gradual evolution over the phylogeny. The values of all these parameters are consistent with the assumptions of parsimony, namely that evolution of a trait is independent of branch length but highly dependent on phylogenetic relationships.

| | Size Dimorph. | Dichromatism | Showiness |
|---|---|---|---|
| Dichromatism | 0.172* | | |
| Showiness | 0.203** | 0.533*** | |
| Spur presence | 0.0174 | 0.00202 | 0.00436 |
| Spur length | 0.0864 | 0.203* | 0.172 |

Table 2.3 Correlations between each of the traits. Values are $r^2$ with *s indicating significance; * p<0.05, ** p<0.01, ***p<0.001.

Sexual dichromatism and male showiness were significantly correlated with sexual size dimorphism (LRSt=5.45, p=0.019 and LRSt=6.56, p=0.010 respectively). The presence of spurs was not correlated with any of the other traits, indicating that this trait is evolving in response to some factor other than that determining male size and plumage colour. It also shows evidence of high phylogenetic constraint, with a λ not significantly different to 1 (LRSt=0.30, p=0.583). In contrast relative spur length

was significantly correlated with sexual dichromatism (LRSt=5.12, p=0.024) although not with male showiness or sexual size dimorphism and again showed high phylogenetic constraint ($\lambda$=1, LRSt=0, p=1).

## Discussion

The phylogenies based on nuclear and mitochondrial sequence data were largely similar to each other and to previously published phylogenies (Kimball et al. 1999; Dimcheff et al. 2002; Bush and Strobeck 2003; Smith et al. 2005). The roulroul partridge has not previously been included in molecular phylogenies and was found to occupy a basal position within the Phasianidae using two different loci and several reconstructions. This is in contrast to a phylogenetic analysis based on morphological characters, which grouped the roulroul with the Old World quails (Dyke et al. 2003). I found strong support for a grouping of the higher pheasants, grey partridge, grouse, turkey, tragopan and monal to the exclusion of the peacock-pheasants, peafowl and argus pheasants, which has been suggested previously but with weaker support (Kimball et al. 1999; Kimball et al. 2001). I also find evidence for a basal position for the junglefowl, which had been suggested by previous mitochondrial phylogenies but with only weak support (Kimball et al. 1999; Kimball et al. 2001; Bush and Strobeck 2003).

Previous studies have given inconsistent and poorly supported placements of the grey partridge (Kimball et al. 1999; Dimcheff et al. 2002; Smith et al. 2005). In contrast I find reasonably strong evidence for a monophyletic grouping of the grey partridge with higher pheasants. The argus pheasant groups monophyletically with the peafowl, which has been weakly supported in only one other study (Kimball et al. 2001). Consistent with some previous phylogenies (Kimball et al. 1999; Dimcheff et al. 2002) but not others (Kimball et al. 2001; Smith et al. 2005), I find evidence for the turkeys being sister to the grouse. Intrageneric relationships in the peacock pheasants (*Polyplectron*) and *Lophura* pheasants are consistent with those found previously (Kimball et al. 2001; Randi et al. 2001). To date relationships within the

higher pheasants have been poorly resolved. Here the Reeve's pheasant is found to group with the common pheasant, as reported previously (Kimball et al. 1999; Kimball et al. 2001) but, in addition, I find the cheer pheasant groups with the eared-pheasant, which has not been previously found.

The phylogeny of the Galliformes has some interesting implications for the evolution of sexually selected traits in these birds. The grouping of the argus pheasants with the peafowl is interesting as both of these taxa show circular "eye-spot" type patterns on the male display feathers. These patterns seem complex and are very restricted among bird taxa, suggesting they many not arise easily. The only other group to show them among the Galliformes are the peacock pheasants, which, in contrast to the previous phylogeny (Kimball et al. 1999), I did not find to group with the peafowl. This suggests that the similarity between the plumage of these birds is either convergent or ancestral to a large portion of the Phasianidae.

Another interesting grouping in terms of plumage colour evolution is that of the cheer pheasants (*Catreus wallichi*) with the eared pheasants (*Crossoptilon* spp.), for which I found strong support in the combined analyses but had not been identified in previous studies. These taxa are both relatively monochromatic but in the eared pheasants both sexes are bright, whereas in the cheer pheasant they are both relatively dull. It has been suggested that cheer pheasants most likely evolved from a dichromatic ancestor, as they possess dichromatic tail feathers that are largely concealed by central, dull coloured feathers, and that this was in response to a change in mating system from polygyny to monogamy (Corder 2004). However if they are most closely related to the eared-pheasant this suggests that the common ancestor of both genera was monochromatic and poses the question of whether it was bright monochromatic with dull monochromatism evolving in the cheer pheasant lineage, or

dull monochromatic with bright monochromatism evolving in the eared-pheasant lineage.

The traditional groupings of the sexually dimorphic pheasants and the sexually monomorphic partridges no longer hold, with the grey partridge (*Perdix perdix*) found to be most closely related to the strikingly dimorphic higher pheasants; and the dimorphic Junglefowl (*Gallus*) a sister group to the partridge-like francolins. This suggests that the level of sexual dimorphism, and hence presumably the level of sexual selection, has undergone several switches during galliform evolution, indicating that sexual dichromatism is fast evolving. This was expected to lead to problems when using parsimony to reconstruct the evolution of this trait. However when modelled under maximum-likelihood the results indicate that the evolution of dichromatism is consistent with many of the assumptions of parsimony. Most of the switches have occurred at the nodes of the phylogeny, with branch length being relatively unimportant, and there is a significant effect of phylogenetic relationships, with closely related taxa tending to have similar levels of dichromatism. This indicates that the best reconstruction with parsimony is likely to represent the true best reconstruction and so makes parsimony a good option for reconstructing ancestral levels of dichromatism in the Galliformes for future studies. A further implication of changes in dichromatism occurring primarily at the nodes of the phylogeny is these changes may be important in the speciation process. This is not necessarily evidence for sexual selection driving speciation because in that case the expectation would be for more sexually dichromatic lineages to be more speciose (Barraclough et al. 1995; Owens et al. 1999). However, it is consistent with divergence in male plumage traits playing a role in species divergence in the Galliformes, as has been suggested previously (Young et al. 1994). This could occur directly, with changes in plumage

colour leading to differences in mate recognition, or indirectly, for example through ecological speciation leading to differences in breeding ecology and so different levels of dichromatism.

Another aspect of sexual dimorphism, size dimorphism, also shows rapid evolution. However in this case its evolution is not consistent with parsimony and appears to have resulted in species-specific adaptation, with little effect of phylogenetic history on its evolution. Although these traits show very different evolutionary patterns they are also correlated. It therefore seems likely that they are both evolving in response to sexual selection with both of these forms of dimorphism being more exaggerated in species that are under stronger sexual selection. Most comparative studies that have considered both size dimorphism and dichromatism have not directly looked for a correlation between these two traits (Hoglund 1989; Owens and Hartley 1998; Figuerola and Green 2000; Dunn et al. 2001; Bennett and Owens 2002). However at least one of these has found both of these traits to be correlated with mating system (Dunn et al. 2001) and therefore it seems reasonable that they should also be correlated with each other if they have both evolved as a result of sexual selection. In addition the correlation between relative spur length and dichromatism suggests that all three of these traits may be evolving in concert in response to sexual selection. The lack of a correlation between relative spur length and sexual size dimorphism however, suggests that these traits may represent alternative strategies adopted on different lineages.

Studies of plumage colour in New World blackbirds (Irwin 1994) and tanagers (Burns 1998) revealed that changes in female colour were more common than changes in male colour and more often associated with changes in dichromatism. This suggested that changes in dichromatism were mainly driven by selection acting on

females rather than males. I find a strong correlation between dichromatism and a measure of male showiness suggesting that this is not the case in the Galliformes and that they may be more similar to the Anseriformes in which changes in male ornamentation were found to be more frequent and the driving factor in dichromatism evolution (Figuerola and Green 2000). Male showiness did show a slightly higher correlation with size dimorphism indicating that it may be a slightly better predictor of sexual selection acting on the males on some lineages but had a lower (non-significant) correlation with relative spur length.

The presence of high showiness compared to dichromatism in the basal lineages may be interesting if it indicates that these lineages arose prior to the evolution of a mechanism for producing sexually dimorphic plumage in the Galliformes and that bright monochromatism is the ancestral state (Kimball and Ligon 1999). This would then explain the apparent sudden appearance of dichromatism in the parsimony reconstructions at the origin of the Phasianidae (Figure 2.5). It may have been this event that then allowed some of the male Phasianidae to evolve such extreme plumage traits as we see today. The basal families are predominantly monochromatic, often with apparently showy female plumage. However the Cracidae has several sexually dimorphic members and the New World quails, although not included in this study, are now considered to be basal in Galliformes (Kimball et al. 1999; Smith et al. 2005), and many exhibit sexually dimorphic plumage traits. Therefore perhaps the lack of dichromatism in some of the basal groups is due to ecological factors and not due to a developmental constraint. This may be consistent with the breeding systems of these birds given that the curassows are tree nesting, not ground nesting like the other Galliformes, and that the megapodes are mound builders and therefore do not require to be camouflaged while incubating.

Studies in ducks (Omland 1997) and passerines (Price and Birch 1996) have found evidence for a small number of gains of dichromatism followed by multiple losses, suggesting that dichromatism can be more easily lost than gained (Wiens 2001). I found a similar pattern when using a reduced number of dichromatism categories (figure 2.5) and a parsimony reconstruction. However, when a larger number of dichromatism categories were used this pattern disappeared, revealing a large number of incremental increases in dichromatism over the phylogeny (figure 2.4). In addition in the maximum likelihood analysis of dichromatism evolution I did not find any evidence for a dominant direction of evolutionary change. Therefore the findings of easier loss than gain of dichromatism in the previous studies may have resulted from the scoring systems they used in which taxa were simply scored as dimorphic or monomorphic.

Spurs also appear to have been a trait that arose early in the evolution of the Phasianidae and were then lost by several lineages within this group. The losses of spurs suggest that there may be a cost to developing or maintaining these structures resulting in their loss when no longer needed. In contrast to the previous study of spurs in the Galliformes (Davidson 1985), I did not find a relationship between the presence of spurs and sexual size dimorphism. However the previous study did not take phylogenetic nonindependence into account, suggesting that the result was due to phylogeny. The presence of spurs showed no correlation with the other traits and appears to be limited by phylogenetic constraint, suggesting that on many lineages the presence of spurs may simply be a historical artefact. However, the continuous measurement of relative male spur length appears to be more informative as a measure of whether spurs are used in female mate choice or male-male competition given the correlation with sexual dichromatism.

Although several members of the Galliformes are very well studied and provide classic examples of sexual selection (Petrie et al. 1991; Petrie 1992; Zuk et al. 1992; Petrie 1994; Mateos and Carranza 1997; Hagelin 2002) there are others that we still know very little about in terms of their mating system and life history characters. This makes it difficult to carry out comparative studies of these birds. It would be interesting to know, for example, if the traits I have investigated here are correlated with the mating system or levels of extra-pair paternity, as would be expected if they had indeed evolved in response to sexual selection.

# Chapter 3

# Pigmentation gene evolution in relation to sexual selection in the galliform birds


## Abstract

**The extravagant plumage traits of male birds are a favourite example of sexual selection. However, to date the units that selection is acting upon, the genes themselves, have been seen as a "black box". Here I sequence four pigmentation genes and two additional loci as controls in an array of galliform species exhibiting a range of degrees of sexual dimorphism. The rate of evolutionary change at these loci is estimated using the ratio of the rate of nonsynonymous substitution (dN) to the rate of synonymous substitution (dS). Lineage-specific dN/dS ratios are then compared to periods of intense or relaxed sexual selection in galliform evolution, using sexual dichromatism as the main index of sexual selection. For one of the loci, the melanocortin-1-receptor locus (*MC1R*), I find a higher rate of evolutionary change on lineages with higher levels of sexual dichromatism, suggesting that sexual selection is driving change at *MC1R*. This is consistent with previous work on colour polymorphisms and suggests that *MC1R* may be a key target for selection acting on plumage colour. The pattern of selection at *MC1R* seems to be consistent with continuous or cyclical evolution of traits and preferences, while two of the other loci (*DCT* and *TYRP1*) show some evidence of increased selective constraint associated with higher sexual selection.**

## Introduction

The extravagant plumage traits of many male birds are a classic example of sexual selection (Darwin 1871; Hamilton and Zuk 1982). Many empirical (Petrie et al. 1991; Zuk et al. 1992; Owens and Hartley 1998; Dunn et al. 2001) and theoretical (Kirkpatrick 1982; Mead and Arnold 2004) studies have greatly improved our understanding of how these apparently costly secondary sexual traits can arise and be maintained. However, the proximate mechanisms linking selection to the resulting male plumage traits have largely been treated as a "black box". An understanding of the genes involved would allow us to fill this gap and investigate the individual units that selection is acting upon.

There are many hypotheses about sexual selection and how it acts to bring about the traits seen in nature. Many of these are hard to discern using conventional comparative data on the phenotypes of extant taxa (Bennett and Owens 2002). Although neither few-locus nor quantitative genetic models make specific predictions about the accumulation of mutations at a single locus, the evolutionary scenarios produced by different quantitative genetic models can be investigated at the single locus level (Mead and Arnold 2004). For example unstable or "runaway" evolution of ornaments, in which a stable level of ornament elaboration is not reached until counteracted by natural selection or loss of genetic variance on which to act, is possible under almost all sexual selection scenarios (Mead and Arnold 2004). This would be expected to result in rapid change at a locus over a short time-scale during ornament elaboration, perhaps followed either by stasis, if the adaptive potential of the locus has been exhausted, or by a more modest level of change as new adaptive mutations arise. The alternative to this scenario is that sexual selection can lead to a stable outcome or equilibrium. Under most "good-genes" models this equilibrium is a

single point (Mead and Arnold 2004). This would be characterised by an elevated rate of change at a locus, although perhaps lower than that seen under a runaway scenario, followed by stasis. Equilibria in the form of lines or cycles have been found under Fisherian, sexual conflict and some "good-genes" scenarios (Iwasa and Pomiankowski 1995; Iwasa and Pomiankowski 1999; Houle and Kondrashov 2002; Mead and Arnold 2004). These would lead to gradual but prolonged or continual change at a locus.

Most "good-genes" models of sexual selection rely on mutation-selection balance in order to maintain genetic variance in a trait under sexual selection (Iwasa and Pomiankowski 1991; Andersson 1994; Pomiankowski and Møller 1995; Rowe and Houle 1996; Houle and Kondrashov 2002). This means that we would expect to find sexual selection acting to remove mutations at loci that may have an effect on the phenotype of the trait. For example the genic capture hypothesis proposes that genetic variance in sexually selected traits is maintained by condition dependence of the sexually selected trait and high genetic variance for condition (Rowe and Houle 1996; Tomkins et al. 2004). This suggests that a large number of loci throughout the genome have an impact on condition and even slightly deleterious mutations at any of these loci would be expected to have a detectable effect on a sexually selected trait and so be purged from the population. Therefore we would predict to find higher levels of purifying selection acting at a large number of loci in taxa with higher levels of sexual selection compared to those with lower levels of sexual selection.

In what could perhaps be seen as an extension of this idea are models that predict an increased rate of mutation in taxa with higher levels of sexual selection (Møller and Cuervo 2003). This could be due to higher levels of sperm competition leading to more meiosis and so a higher rate of mutation (Bartosch-Härlid et al. 2003)

or to specific mutator alleles. These would act to maintain genetic variation in sexually selected traits with sexual selection acting in opposition to remove deleterious mutations. Once a reasonable sample of molecular data is available for a range of taxa it will be relatively simple to test the prediction that taxa with stronger sexual selection will have higher rates of mutation.

Adaptive evolution can be detected by comparing the rate of nonsynonymous (amino acid) to synonymous (silent) substitution (dN/dS), with dN/dS>1 taken to indicate positive selection. Previous evidence of sexual selection at the molecular level has come from studies of proteins closely related to primary sexual traits (proteins with direct roles in reproduction), including accessory gland proteins in *Drosophila* (Kern et al. 2004), mammalian sperm and seminal fluid proteins (Torgerson et al. 2002; Jensen-Seaman and Li 2003; Dorus et al. 2004; Glassey and Civetta 2004; Bustamante et al. 2005), and sea urchin gamete recognition proteins (Geyer and Palumbi 2003; McCartney and Lessios 2004). These have all been found to be rapidly evolving, and frequently show dN/dS>1. However, in only one case is there evidence that it is indeed sexual selection that is driving the high rate of evolution, in the form of a correlation between this and the level of intrasexual competition (Dorus et al. 2004). The above traits are largely only expressed in one sex and unconstrained by natural selection except perhaps by the reproductive proteins of the opposite sex, which could be equally fast evolving (Swanson et al. 2001b).

In contrast, genes controlling secondary sexual traits are likely to be expressed in both sexes, relying on gene regulation to produce differential effects in either sex, and will be constrained by the functional demands of the animal. In the case of birds, the feathers must still function for insulation and flight and the females and juveniles must remain cryptic. In addition, unlike primary sexual traits, which will be expected

to be controlled by similar genes across species, we have no a priori expectation that the same locus will be involved in producing secondary sexual traits across many species. This is particularly clear when we consider the diversity of secondary sexual plumage traits and colours seen in birds. If the same genes are not selected across different lineages this will also severely reduce our ability to detect sexual selection acting at any individual locus. Therefore we may expect sexual selection to be harder to detect in genes controlling secondary sexual traits as compared to primary sexual traits and it seems unlikely that ratios of dN to dS in excess of 1 will occur.

In this study I investigate the effect of sexual selection on a set of genes involved in melanin synthesis in the galliform birds (pheasants, partridges and allies). These birds show extraordinary diversity in ornamental male plumage colour, and provide some of the classic empirical demonstrations that plumage colour is important for female choice (Petrie et al. 1991; Zuk et al. 1992) or male-male competition (Mateos and Carranza 1997). Plumage colour in this group of birds is largely due to melanins and fine feather structures, while carotenoids are generally considered not to be used. In addition, the structurally coloured feathers that have been studied in these birds have all involved ordered arrays of melanosomes (the melanin containing organelles) that produce colour by differentially scattering visible wavelengths of light (Zi 2003; Prum 2006).

Three of the genes I consider here code for enzymes in the melanin synthesis pathway. The first of these is the tyrosinase gene (*TYR*), which encodes a key enzyme in melanin synthesis and is required for the production of both pheomelanin (paler yellow/reddish-brown melanin) and eumelanin (darker black/brown) (Sturm et al. 2001). This gene has been isolated in chickens (Mochii et al. 1992), and known mutations result in albinism (Tobita-Teramoto et al. 2000) or white plumage (Chang

et al. 2006). The other two are the tyrosinase-related protein-1 (TYRP1) and the tyrosinase-related protein-2 (also know as DOPAchrome tautomerase, DCT). These are both required for the production of eumelanin but not pheomelanin and have both been identified in chickens (April et al. 1998a; April et al. 1998b). Although mutations with effects on pigmentation in birds have yet to be found, in mice a loss-of-function mutation at the *TYRP1* locus produces a brown coat (Zdarsky et al. 1990), and a mutation at the *DCT* locus, which reduces but does not abolish enzyme activity, produces a "slaty" phenotype (Budd and Jackson 1995).

The other pigmentation gene included here codes for the melanocortin-1-receptor (MC1R). This is a 7-transmembrane G-protein-coupled receptor that sits in the membrane of the melanin producing cell (melanocyte) (Garcia-Borron et al. 2005). Activation of this receptor stimulates melanogenesis and switches it from synthesis of pheomelanin to synthesis of eumelanin. *MC1R* has been identified in chickens (Takeuchi et al. 1996) and several mutations with effects of feather colour have been identified in chickens (Takeuchi et al. 1998; Kerje et al. 2003; Ling et al. 2003) and other bird species (Theron et al. 2001; Mundy et al. 2004).

I also investigate the evolution of the agouti related protein locus (*AGRP*). The melanocortin receptors are unique among G-protein-coupled receptors in having endogenous antagonists (Garcia-Borron et al. 2005). In mammals the antagonist of the MC1R is the agouti signalling protein (ASIP) and AGRP acts as an antagonist for melanocortin receptors in the central nervous system where it is involved in control of food intake (Schwartz 2000). AGRP has also been identified as having this role in the central nervous system of birds (Boswell et al. 2002). However until recently *ASIP* was unknown in birds and *AGRP* was found to be expressed in the skin (Takeuchi et al. 2000) and so postulated to also be involved in pigmentation in birds (Boswell and

Takeuchi 2005). With the recent identification of *ASIP* in birds (Klovins and Schiöth 2005and see chapter 5), it now seems less likely that *AGRP* has any role in pigmentation, and therefore it will primarily be treated as a control in this study. An additional control in the form of the Cytochrome *b* gene (*CYTB*) is also included.

*TYRP1* is found on the Z chromosome in chickens and therefore highly likely to be sex-linked across the Galliformes. All other nuclear loci investigated were autosomal. Sex-linked loci have been suggested to be particularly likely to be involved in the production of secondary sexual ornamentation (Reinhold 1998; Sætre et al. 2003; Kirkpatrick and Hall 2004; Albert and Otto 2005). In addition they are most likely to show an increased rate of mutation in species with higher sperm competition, as at any one time 2/3 of Z chromosomes will be found in males (the homogametic sex) (Bartosch-Härlid et al. 2003).

## Methods

### Laboratory Methods

DNA was extracted from soft tissue samples from 36 species of galliform bird (see appendix 2 for a full list of taxa and samples). Partial coding sequence was obtained for 5 pigmentation genes: *MC1R, TYR, TYRP1* and *DCT;* and two additional loci: *AGRP* and *CYTB,* as previously described (Chapter 2).

### Analysis of ω in relation to sexual dichromatism and presence of spurs

dN/dS (ω) values were obtained for each locus individually by maximum likelihood, using a codon-based substitution model in PAML version 3.15 (Yang 1997). Sexual plumage dichromatism was scored from 0 to 6 and its evolution reconstructed over the galliform phylogeny using MacClade 4 (Maddison and Maddison 2000) as described in chapter 2. As mentioned previously (chapter 2), this resulted in some branches with states that could not be resolved unequivocally. These were resolved using equivocal cycling. Using the dichromatism values as 7 branch categories, ω values were calculated for each category using a 7-ratio model in PAML (figure 3.1). Heterogeneity in ω among these lineages was tested using likelihood ratio tests comparing these models to null models with a single ω across the phylogeny. The phylogeny used for this analysis was the Bayesian MCMCMC phylogeny using all of the above loci and *AGRP* introns, as described in Chapter 2.

The character states on the branches leading to the curassow and megapode could not be reconstructed with as much confidence due to the long branch lengths and sparse sampling of taxa. Therefore after an initial test including these taxa they were excluded from all further analyses. The relationship between sexual plumage dichromatism and ω was tested using a linear regression.

To determine if ω was responding to a constant level of dichromatism or to changes in dichromatism, the analysis was also performed using branch groupings corresponding to the level of change in dichromatism. This was either scored as 7 categories (increase of 1, 2 or 3, no change or decrease of 1, 2 or 3) or as 3 categories (increase, no change or decrease). In addition, the analysis was also performed using a score of male showiness (male colour compared to juvenile colour) reconstructed using MacClade (see chapter 2 for details).

This method of analysis was also used to compare ω on branches with and without spurs (2 branch categories) for each of the loci.

**Alternative genus-level analysis**

The above analysis is powerful in that it uses the data for each of the loci over the whole phylogeny, however it relies on a single phylogeny and a single parsimony-based character reconstruction. Repeating that analysis with different phylogenies would have been labour intensive and many of the alternative phylogenies yielded large numbers of equivocal branches when using maximum-parsimony character reconstruction. In order to overcome this problem an alternative method was used in which ω values and dichromatism scores were compared at the genus level to look for correlations between these measures. Free-ratio models were implemented in PAML, which estimate a ω value for every branch on the phylogeny. Dichromatism scores and ω values at the genus level were analysed as traits in the comparative analysis program, "Continuous" (Pagel 1997), which corrects for any effect of phylogenetic non-independence. Dichromatism values were averaged at the genus level and mean ω values (weighted by branch length) were calculated for each genus (including the branch leading to the common ancestor of all species within the genus and all branches leading from this level to the tips). To check this method, mean ω ratios for

these groupings were also calculated in PAML for the *MC1R* data set and the results were very similar. The analysis was done at the generic level to ensure that enough information was available to reliably calculate ω; values based on three or fewer nucleotide changes were excluded from the analysis since these do not provide enough information.

To check that the phylogeny did not affect the results, the *MC1R* data set was also analysed using six additional phylogenies with noticeably different topologies arbitrarily sampled from within the Bayesian tree sample, and also analysed using the consensus tree from the analysis of *AGRP* introns and *CYTB* only, to check that using a phylogeny based on the pigmentation gene coding sequences did not bias the results.

This analysis was also performed comparing ω to male showiness, presence of spurs, relative male spur length and sexual size dimorphism (see chapter 2 for scoring methodology). Sexual size dimorphism and relative spur length could only be analysed using this methodology, as these were measured on a continuous scale and so could not be used to discretely categorise branches as in the previous analysis methodology. In addition, combined dS at all 5 nuclear loci was compared to sexual dichromatism to test for an effect of sexual selection on background mutation rate. As *TYRP1* is a sex linked locus and therefore may be most likely to show a relationship between mutation and sexual selection, dS at this locus only was also compared to the presence of spurs, relative male spur length, the level of sexual dichromatism, and sexual size dimorphism.

**Sliding window analysis of *MC1R* ω in relation of sexual dichromatism**

In order to find out if the signature of sexual selection at *MC1R* was due to particular regions of the coding sequence, it was divided into portions and each analysed individually using the 7-ratio model as described above with regression lines fitted to

the seven points. Preliminary analysis revealed that regions of at least 285bp were required for the LR tests to have enough power to detect differences in ω between the dichromatism categories. Sliding window analyses were then performed first with a window of 297bp and an offset of 33bp between datasets, and then with a window of 396bp and an offset of 33bp. These compound input datasets were created using the program ADS Gene Shoogle v1.0 (Smith 2006).

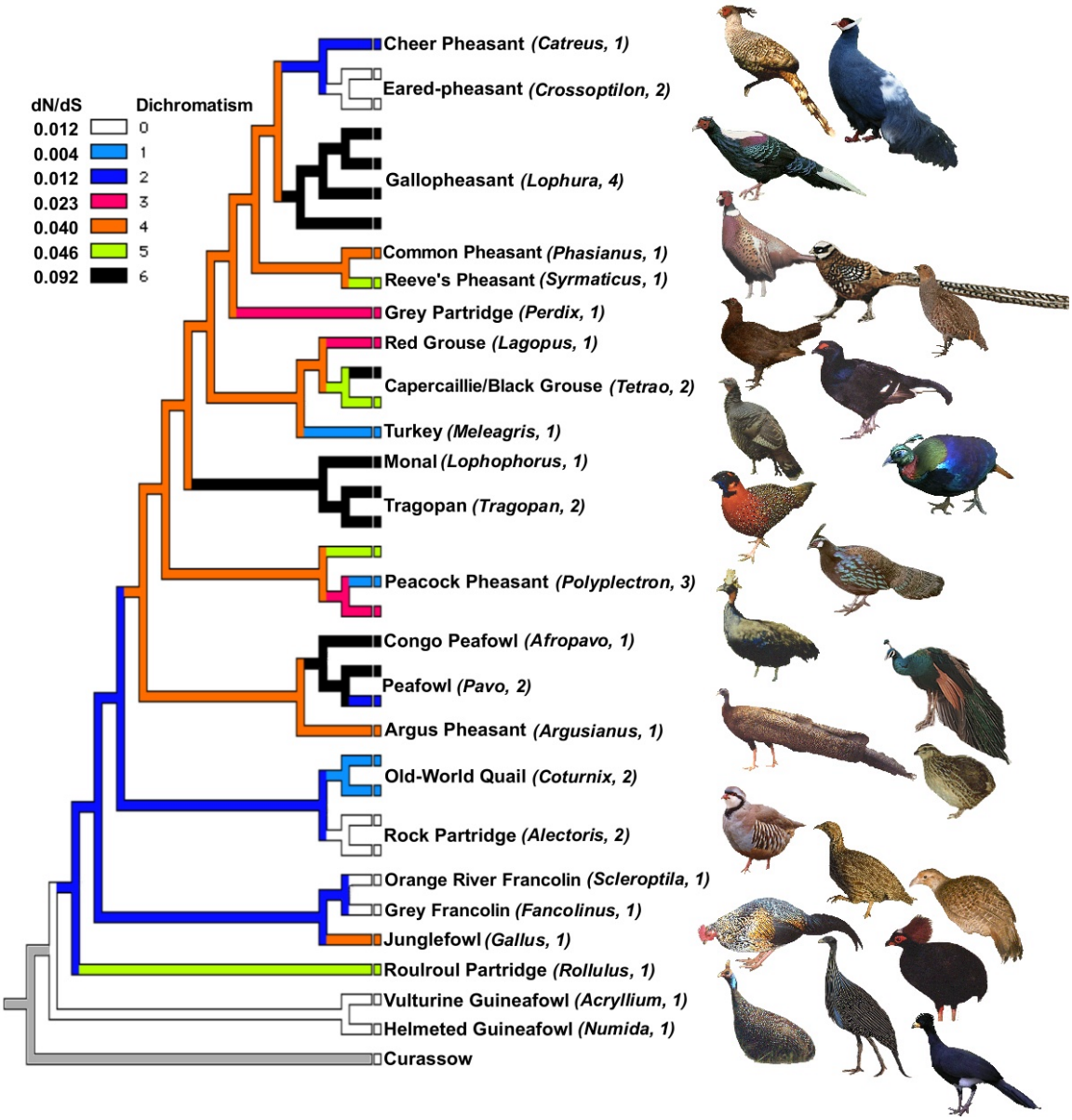



Figure 3.1. Reconstruction of plumage dichromatism evolution on the phylogeny of the galliform species in this study, with mean ω (dN/dS) at *MC1R* estimated for each dichromatism category. Grey branches were not included in the analysis. Genera are listed as: common name (scientific name, number of species sampled). An image of a male from each genus is shown to the right (Images courtesy of J. Corder, M. Anderson and A. Smith).

## Results

### Analysis of ω in relation to sexual dichromatism and presence of spurs

At *MC1R,* a model with ω differing between dichromatism categories was found to fit the data significantly better than a model with a single ω for all branches (LRSt=15.5, df= 6, p=0.0164). This indicates that the level of functional change at *MC1R* can be linked to the level of sexual plumage dichromatism (figure 3.1). Furthermore a positive regression line was found to fit the values with a significant proportion of the variation in ω explained by the level of dichromatism ($r^2$= 0.684, p=0.0218). The ω value for the dichromatism = 0 category appears to be an outlier on this plot (figure 3.2), which may partly be influenced by the branches leading to the outgroups, the curassow and megapode. These long branches were reconstructed as having zero dichromatism, however it is far from certain that the ancestor was sexually monochromatic (see chapter 1 for discussion). Therefore these branches were removed and the analysis repeated. This strengthened the fit of the dichromatism branch model and strengthened fit of the regression (figure 3.3). These results were also significant under a Bonferroni correction for multiple tests (corrected α-level = 0.0083 for 6 tests). When this analysis was performed using the other loci no significant LRSts were found and there were no significant regressions between dichromatism and ω (figure 3.3), although for *DCT* there was an inverse relationship, which was approaching significance ($r^2$ = 0.563, p=0.0519).

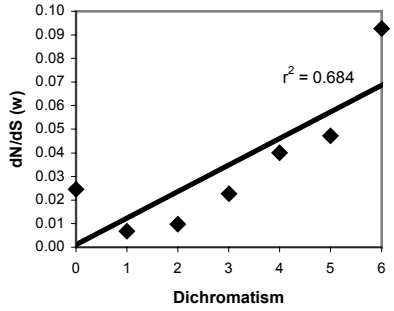



Figure 3.2 Linear regression between sexual plumage dichromatism and ω at *MC1R* for the different dichromatism categories, with the outgroups (curassow and megapode) included.

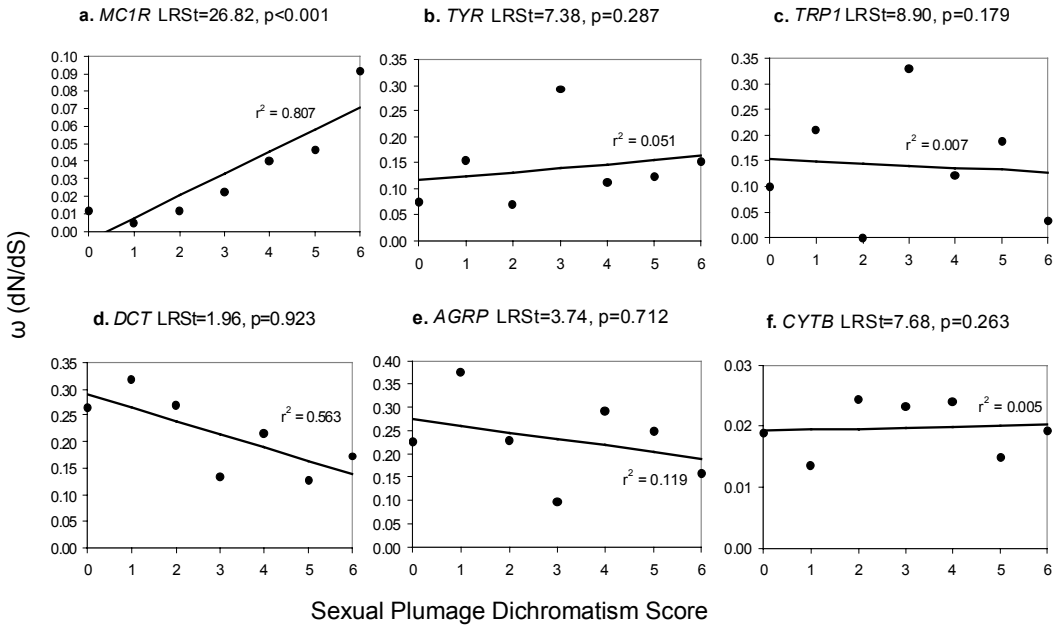



Figure 3.3 Relationships between sexual plumage dichromatism and ω for different categories of dichromatism. Likelihood ratio statistics (df=6) and their significance values for the heterogeneity test in PAML are given. Significance values for the regressions are not shown. For clarity, y axes are on different scales.

When ω at *MC1R* was modelled over the phylogeny with branch categories corresponding to the six male "showiness" categories (none of the sampled taxa had showiness = 0), these groupings did not significantly explain the variation in ω (LRSt=7.72, df=5, p=1.72). Although ω in the showiness = 6 category was higher than that of the other categories, there was no significant relationship between ω and showiness ($r^2$=0.329, p=0.234).

Seven "change in dichromatism" categories (-3,-2,-1,0,+1,+2,+3) were found to significantly explain some of the variation in ω at *MCIR* (LRSt=13.7, df=6, p=0.0329). However this appeared to be strongly influenced by a large ω value for the +1 category (figure 3.4) and was no longer significant when the change was reduced to three categories (decrease, no change, increase) (LRSt=3.44, df=2, p=0.179).

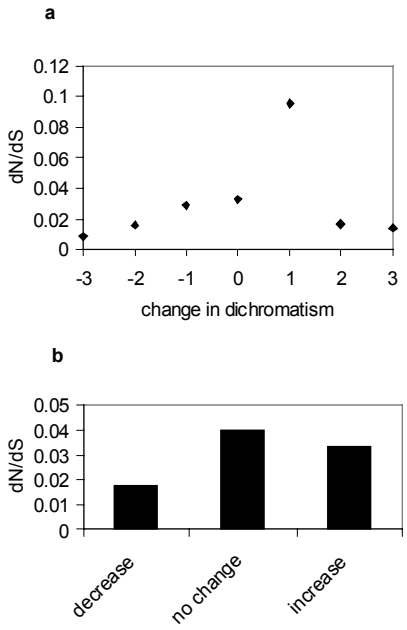



Figure 3.4 Relationships between the change in sexual plumage dichromatism and ω at *MC1R* for 7 categories of the level of change (**a**) and for 3 categories of the type of change (**b**).

At *TYRP1* branches with spurs were found to have significantly lower ω values than those without (figure 3.5), although this is not significant when correcting for multiple tests. No differences between these branch categories were found for any of the other loci. Looking at dN and dS separately at *TYRP1,* it appears that dS is similar in both branch categories, while dN appears to be lower on branches with spurs (data not shown). Therefore it appears that the decreased ω is due to decreased dN on branches with spurs rather than increased dS, suggesting that this is not due to differences in background mutation rate on the different branches.

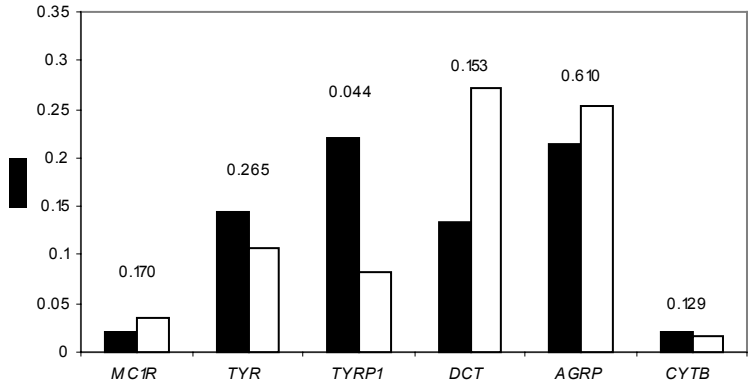



Figure 3.5 Relationship between the presence of spurs and average ω for lineages grouped as having spurs (white bars) or no spurs (black bars). Significance values are given above the bars for each of the loci (df=1).

**Alternative genus-level analysis**

Comparing ω values and dichromatism at the genus level in the program Continuous, there was significant covariance between dichromatism and ω at *MC1R* (LRSt=8.5, df=1, p=0.004). I did not find significant covariance between ω and dichromatism for any of the other loci studied (figure 3.6). These results were robust to changes in phylogeny; a significant association between *MC1R* ω and dichromatism was found with seven alternative phylogenies (LRSt ≥ 6.2, df =1, p ≤ 0.013). Using this method there was also a significant correlation between male showiness scores and ω at *MC1R* ($r^2$=0.23, p=0.02).

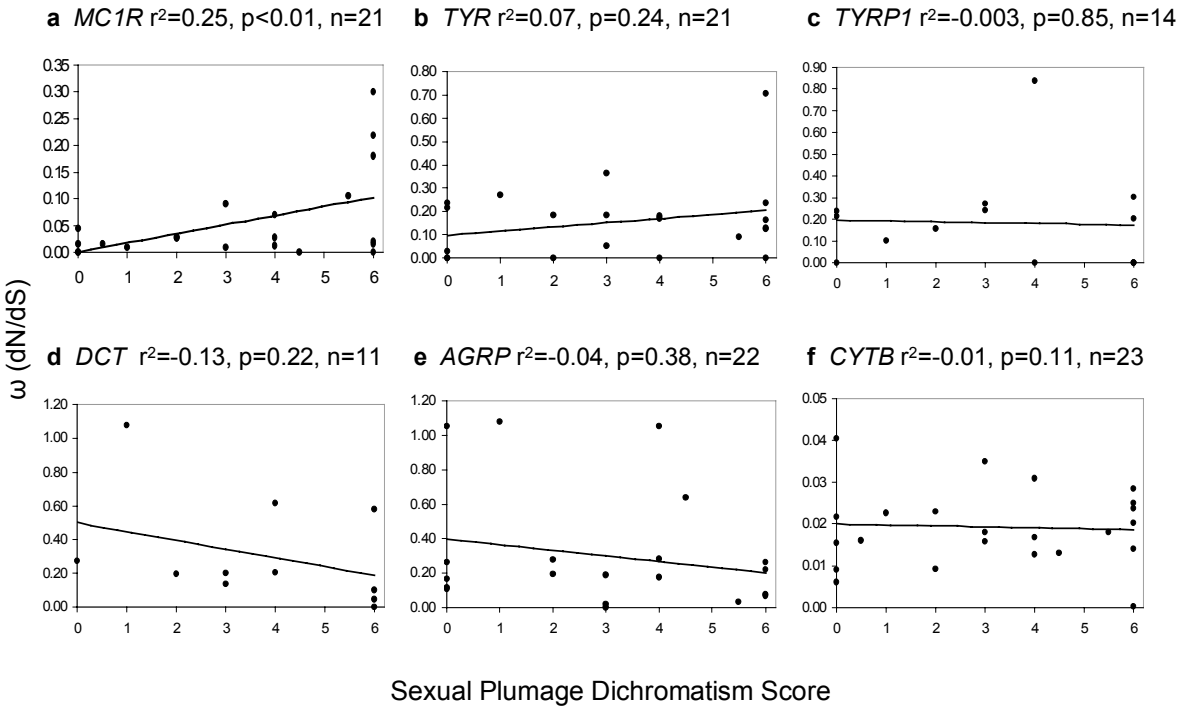



Figure 3.6 Relationships between ω and sexual plumage dichromatism. Each point represents one genus with average dichromatism for each genus and ω averaged for branches leading to the genus level and terminal branches within the genus. Correlations were calculated using Continuous. For clarity, y axes are on different scales.

A comparison between ω and relative male spur length at the genus level produced a similar result at *TYRP1* to the analysis of spur presence or absence over the whole phylogeny (figure 3.7). Significant inverse covariance was found between relative male spur length and ω at this locus (LRSt=4.95, df=1, p=0.026). dS at TYRP1 was not correlated with relative male spur length ($r^2$<0.001, p=0.94), again suggesting that the relationship between ω at TYRP1 and spurs is due to decreased dN not increased dS and therefore not due to differences in background mutation rate. None of the other loci showed significant correlations between ω and relative spur length, although at *MC1R* there was a slight tendency towards a positive relationship between ω and spur length (LRSt=2.86, df=1, p=0.088), which is most likely explained by the correlation between dichromatism and relative male spur length (see chapter 2).

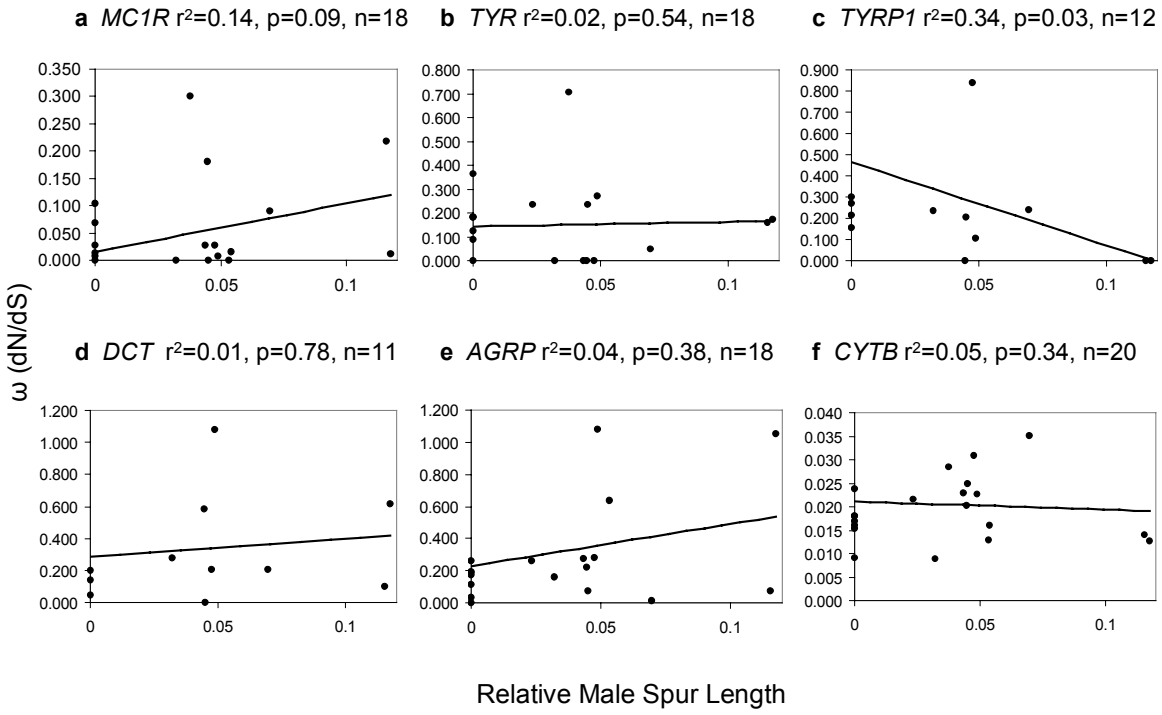



Figure 3.7 Relationships between ω and relative male spur length (maximum male spur length/ male wing length). Correlations were calculated using Continuous. For clarity, y axes are on different scales.

No relationships were found between ω at any of the loci and size dimorphism (LRSt ≤ 0.8, df=1, p≥ 0.36) or the presence of spurs (LRSt≤ 2.8, df=1, p≥ 0.10, including *TYRP1*, LRSt=0.712, df=1, p=0.399). Similarly there was no correlation between dS and dichromatism in a combined analysis of all five nuclear loci ($r^2$=0.001, p=0.88), suggesting there are no differences in the level of background mutation between these branches. *TYRP1* is sex-linked and therefore is the most likely candidate for showing differences in mutation rate associated with different levels of sexual selection but showed no relationship between dS and the presence of spurs, size dimorphism or dichromatism (LRSt≤ 1.8, df=1, p≥ 0.18).

**Sliding window analysis of *MC1R* ω in relation of sexual dichromatism**

The initial analysis with a window size of 297bp revealed two peaks of significant heterogeneity in ω between dichromatism categories (figure 3.8a, LRSt values), which

appear to correspond to the second transmembrane region and the second extracellular loop/ fifth transmembrane region. These regions also show peaks in average ω (figure 3.8b), supporting the hypothesis that the observed pattern is due to increased adaptive evolution not constraint on some branches. However these regions also have some of the lowest regression statistics for dichromatism and ω, suggesting that this heterogeneity is not always in the predicted direction. Lack of agreement between the heterogeneity and the regression results may partly be due to a high level of stochastic variation due to the relatively small window size. The only region where the regression and the heterogeneity result appear to be in agreement was at the 3' end, where both showed low values indicating that this region is less important in producing the observed signal. However, at this end there is a large peak in ω for the highest dichromatism category, suggesting that even this region may be important.

Using a larger window of 396bp did result in increased agreement between the regression statistic and the likelihood ratio statistic for the heterogeneity test. Both appeared to show three peaks corresponding roughly to the third and fourth transmembrane regions and the third intracellular loop region (although the large window size makes it harder to pin the results to a particular region). Although again both the 3' and the 5' ends showed peaks of ω in the dichromatism = 6 category.

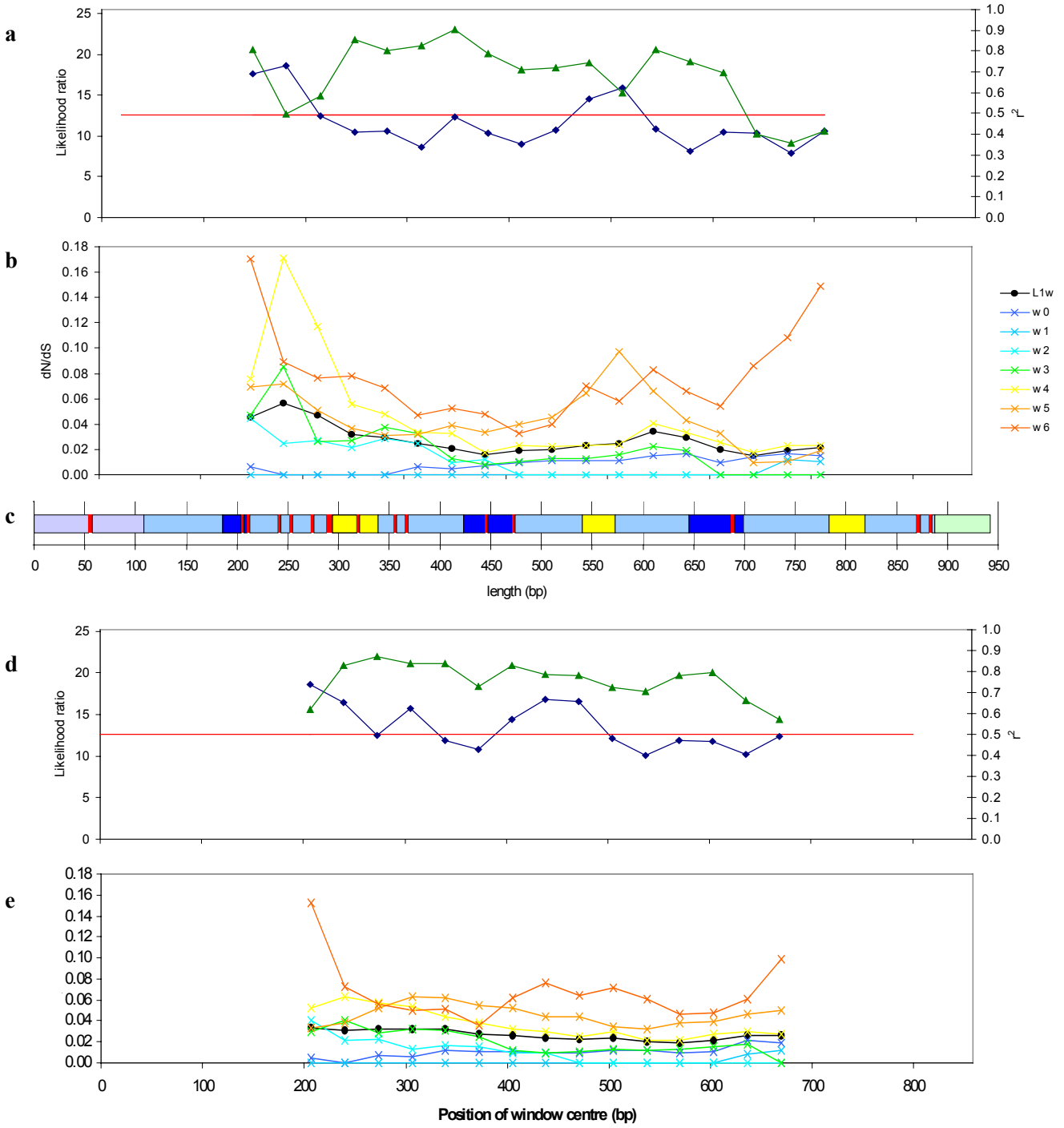



Figure 3.8 Sliding window analysis of the relationship between ω and dichromatism at *MC1R*. Window sizes of 297 (**a**, **b**) and 396 (**d**, **e**) were used. **a** and **d**, likelihood ratio statistics (df=6) (blue) from the heterogeneity test and the $r^2$ values (green) for the regressions of ω on dichromatism. Red lines indicate the 95% significance threshold. **b** and **e**, ω values for the average of all branches (black) and for each dichromatism branch category (w0 to w6, key to colours shown to right of **b**). **c**, schematic representation of the structure of *MC1R*, showing the N-terminus (purple), transmembrane regions (pale blue), intracellular loops (dark blue), extracellular loops (yellow) and C-terminus (green). MC1R sequence variants associated with colour change in birds or mammals are shown in red (see figure 1.3 for details of mutations etc.). The position of the window centre is labelled from the sequenced region, with position 1 corresponding to position 64 of the full *MC1R* coding sequence shown in **c** (numbered according to chicken *MC1R*)

## Discussion

There is a strong and significant pattern of higher dN/dS (ω) for *MC1R* on lineages with higher dichromatism, which is not found at any of the other loci investigated. The absence of this pattern at any of the other loci investigated suggests that the result at *MC1R* cannot be due to non-selective confounding factors such as time since divergence or population size, which have also been shown to affect ω values (Rocha et al. 2006). This pattern is found with two separate methods of analysis, which utilise different character reconstruction methodologies, and is robust to changes in phylogeny. The higher ω on lineages with higher dichromatism are likely to indicate a higher level of positive selection acting on the *MC1R* pigmentation gene, although an increased rate of change due to decreased constraint with neutral drift cannot be ruled out, as the ω values do not exceed one (Yang and Bielawski 2000). It seems that the most likely explanation for this pattern is positive selection acting on the *MC1R* in temporal bursts, and/or in restricted parts of the gene, masked by the background of strong constraint at *MC1R* (average ω = 0.03). The critical function of *MC1R* in regulating melanogenesis (Robbins et al. 1993) makes it very unlikely that the level of functional constraint fluctuates at this locus. In addition, the sliding window analysis indicates that the pattern is strongest for regions of the gene with the highest average ω, indicating that change is relatively more important than constraint. Therefore it seems that the pattern is not due to increased constraint on the monochromatic lineages, as might result from increased natural selection pressure on males or sexual selection pressure on females.

Involvement of *MC1R* in the evolution of dichromatism is consistent with the finding that some *MC1R* alleles have differential effects on colouration among the sexes in domestic chickens (Kerje et al. 2003). Evidence that some of the changes

seen at *MC1R* during galliform evolution have important functional consequences is found in two taxa (*Polyplectron* and *Crax*) with medium to dark colouration that have a glutamate to lysine change at amino acid position 92, which is known to be associated with increased melanin deposition in chickens, bananaquits and mice (Robbins et al. 1993; Takeuchi et al. 1996; Theron et al. 2001). In addition one of the regions identified in the sliding window analysis as contributing most strongly to the pattern of variation in ω at *MC1R* is the second transmembrane region, which is where a large number of the known natural mutations in other taxa are clustered (Garcia-Borron et al. 2005; Mundy 2005). However this analysis also indicates that the whole of the sequenced region of *MC1R* was involved in producing the observed pattern of high ω on lineages with high dichromatism. This may be because the known functional mutations are those that produce the largest phenotypic effects and so are clustered in the most important functional regions of *MC1R*, while mutations in the other regions may be responsible for more subtle colour changes.

Sexual dichromatism is widely accepted as a measure of pre-mating sexual selection (Sorci et al. 1998; McLain et al. 1999; Prinzing et al. 2002; Morrow and Pitcher 2003) and several studies have found correlations with other measures of sexual selection such as mating system (Andersson 1994; Figuerola and Green 2000; Dunn et al. 2001) or extra-pair paternity (Owens and Hartley 1998; Bennett and Owens 2002). I therefore propose that the increased ω at *MC1R* in lineages with higher dichromatism is due to sexual selection acting at this locus. However, dichromatism could be associated with other factors such as differential habitat use by males and females, or loss of showy plumage in females due to predation pressure (Irwin 1994; Kimball and Ligon 1999). To counter this an alternative measure of male showiness (male plumage colour compared to juvenile plumage colour) was also

used. While these scores were very similar to the dichromatism scores for most taxa and were significantly correlated with ω at *MC1R* in the analysis at the genus level, they did not show a significant relation to ω at *MC1R* in the main analysis. This may be because the main analysis relies on a parsimony reconstruction of character evolution and showiness was not as well modelled by parsimony as dichromatism was (see chapter 2). In addition, this measure of showiness has not been widely used or tested and may be a poor measure of sexual selection, perhaps influenced by different selection pressures acting on the juveniles or factors other than sexual selection acting on adult male plumage colour. Alternatively it could be that the increased rate of evolution at *MC1R* on dichromatic lineages is due to increased female crypsis, not increased male showiness (Irwin 1994; Kimball and Ligon 1999). However this is inconsistent with the finding that ω is not related to change in dichromatism, only the absolute level of dichromatism; male plumage colour frequently changes dramatically between equally dichromatic lineages whereas female plumage colour tends to remain very similar.

One surprising feature of these results is that, despite the enormous diversity in male–female colour differences in galliform birds generated since they diversified over 50 million years ago (Tuinen and Dyke 2004; Pereira and Baker 2006), a proportion of this variation has a common genetic basis. A role of *MC1R*, but not the other pigmentation loci examined, in the evolution of dichromatism across the Galliformes is reminiscent of its repeated involvement in the evolution of melanic polymorphisms in birds and other vertebrates (Takeuchi et al. 1996; Theron et al. 2001; Eizirik et al. 2003; Hoekstra et al. 2004; Mundy et al. 2004; Rosenblum et al. 2004) and strongly suggests that there are particular characteristics of this locus that make it a target of directional selection. One possibility is that amino acid

substitutions at *MC1R* are generally subject to a low cost because of few, if any, negative pleiotropic effects at this locus (Mundy 2005).

That I have found a detectable signature of selection over long time-scales suggests that multiple amino acid substitutions at *MC1R* with effects on plumage phenotype have occurred during the diversification of the galliform birds. In addition this increased rate of substitution seems to continue once a high level of dichromatism has been attained since it is not only associated with increases in the level of dichromatism. This seems to be highly suggestive of continuous or cyclical evolution in ornaments and preferences (Pomiankowski and Iwasa 1998; Mead and Arnold 2004). Furthermore, point substitutions at *MC1R* are associated with large-scale variation in melanin distribution in several bird species (Takeuchi et al. 1998; Theron et al. 2001; Mundy et al. 2004) but directional selection for mutations of large phenotypic effect under unvarying sexual selection would not be detectable when averaged over long periods of evolutionary time. Therefore under continuous or cyclical evolution novel *MC1R* alleles of moderate to large phenotypic effect may have become fixed during one regime of sexual selection, with other alleles selected under subsequent regimes. One problem with this argument is that there may have been ascertainment bias in the detection of *MC1R* mutations, as mentioned above, in that those with the largest phenotypic effects have tended to be identified most readily. However, the idea of continuous evolution is supported by the fact that Galliformes are generally regarded as prominent examples of rapid plumage change under sexual selection (Andersson 1994; Young et al. 1994).

Under most "good-genes" models of sexual selection a decrease in ω values on branches with higher sexual selection is expected due to mutation-selection balance (Iwasa and Pomiankowski 1991; Andersson 1994; Pomiankowski and Møller 1995;

Rowe and Houle 1996; Houle and Kondrashov 2002). This is clearly not the case for *MC1R,* however there is some evidence for this at other loci. At *DCT* there is a relatively strong negative regression between dichromatism and ω, although this is likely to be an over-estimate since the errors in the estimates of ω are not taken into account. However there is also limited power to detect selection patterns at *DCT* given the relatively short length of coding sequence analysed (233bp). Similarly, at *TYRP1* there was evidence for decreased ω on lineages with spurs relative to those without and on lineages with longer spurs relative to shorter spurs, suggesting higher constraint on lineages with stronger sexual selection. These results do not stand up to Bonferroni corrections for multiple testing, although this correction is known to be highly conservative. In addition the result concerning the presence of spurs was not replicated in the genus-level analysis, although this was most likely because of low power due to the short length of sequence (267bp). The finding of similar results with two different measures of spurs and two different analysis methods, does seem to suggest that these results are not simply anomalies. These results are clearly far from conclusive but suggest that "good-genes" sexual selection may be acting to remove deleterious mutations, which would worth testing further with a wider screen of more loci.

I have found no evidence for models that predict a genome-wide increase in the level of background mutation in lineages under strong sexual selection (Møller and Cuervo 2003), as there was no correlation between dS and dichromatism in a combined analysis of all five nuclear loci. Even at *TYRP1*, which is on the Z chromosome and so may be expected to show the strongest effects of sperm competition on mutation rate, given that Z chromosomes will spend more time in males than females (Bartosch-Härlid et al. 2003), there was no correlation between dS

and any of the measures of sexual selection. It has also been widely predicted that sex-linked loci particularly on the Z chromosome should be more easily co-opted by sexual selection (Kirkpatrick and Hall 2004; Albert and Otto 2005) and indeed there is some empirical evidence for this (Reinhold 1998; Sætre et al. 2003). However in this study *TYRP1* is the only sex-linked locus and does not appear to be under strong sexual selection.

The results presented here suggest that signatures of sexual selection acting on secondary sexual traits are detectable in genetic coding regions, even against a background of strong constraint. This is in contrast to previous findings of sexual selection at the molecular level, which have concentrated on rapidly evolving traits with a direct role in reproduction (Dorus et al. 2004). It will be interesting to see if this finding can be replicated at other loci, or if *MC1R* is unique in being so strongly amenable to sexual selection in a wide range of taxa and over long timescales. The results also support the hypothesis that coding regions are important for generating interspecific phenotypic variation, a phenomenon for which gene regulation is often considered more important (Carroll et al. 2005). This is perhaps particularly surprising in this case given that we are considering traits that are differentially expressed between the sexes.

# Chapter 4

# Population genetics of *MC1R* in the grouse (subfamily Tetraoninae) with a focus on the red grouse (*Lagopus lagopus scoticus*) and black grouse (*Tetrao tetrix*)


## Abstract

**The red grouse and black grouse differ in their mating system, level of sexual plumage dichromatism and male plumage colour. Red grouse are perhaps the most monogamous of grouse species, whilst black grouse are lek breeders. Although the females of these species are similar in colour the males are highly divergent with male black grouse exhibiting striking black plumage while male red grouse are similar in colour to the females but with redder plumage during some seasons. Here I investigate the evolution of *MC1R,* a locus known to be involved in the regulation of black versus red colour, between and within these species. I find evidence for an elevated rate of amino acid substitution between these species, which is suggestive of an adaptive change at this locus. In addition there appears to be a lower level of amino acid substitution within the black grouse suggestive of stronger constraint at *MC1R* in this species.**

## Introduction

The grouse are a large and widespread temperate subfamily within the Phasianidae. In this study I will focus on two species: the black grouse (*Tetrao tetrix*) and the red grouse (*Lagopus lagopus scoticus*). Red grouse are the UK subspecies of willow ptarmigan (*Lagopus lagopus*) and differ from the other subspecies in that they do not undergo a winter moult into white plumage. Black grouse and red grouse are divergent both in mating behaviour and plumage colour. Black grouse are lek breeders with a small number of males securing most of the matings (Rintamaki et al. 1999) although females tend to mate with only one male (Alatalo et al. 1996). Additionally they are strongly sexually dimorphic in plumage, with males exhibiting striking black plumage with a black lyre-shaped tail and contrasting white undertail coverts, whereas the females are cryptic. In contrast the red grouse are largely monogamous. Males secure a territory and usually attract a single female to it, although polygyny (with 2-3 females) occasionally occurs (Hannon and Martin 1992). Males are also involved in accompanying the family and helping to guard the chicks. Red grouse have relatively low levels of sexual dichromatism; during the breeding season they are essentially monochromatic but prior to territory and mate acquisition in the autumn the males moult into brighter, chestnut-coloured plumage.

A small number of capercaillie (*Tetrao urogallus*) individuals were also included in this study. The capercaillie is more closely related to the black grouse than the red grouse (Lucchini et al. 2001; Dimcheff et al. 2002). The male also has dark, almost black, plumage colouration, although it is perhaps less strikingly dichromatic than the black grouse. Capercaillie are much larger than black grouse and also have more pronounced sexual size dimorphism. Breeding is promiscuous or polygynous

(males may occasionally obtain a harem) and males display to attract females, either singly or in leks (Madge and McGowan 2002).

In black grouse female choice seems unrelated to most aspects of male morphology (Alatalo et al. 1991; Rintamaki et al. 1997) and is primarily determined by how close the male's territory is to the centre of the lek (Hovi et al. 1994; Rintamaki et al. 1995; Kokko et al. 1999). Females will, however, use male's tails to assess fighting ability, preferring to mate with males with undamaged tails (Alatalo et al. 1991) particularly when having to choose between peripheral males (Hoglund et al. 1994). In both black grouse and ptarmigan the main ornaments found to be used in mate choice and male-male competition are the combs (Hannon and Eason 1995; Hannon and Dobush 1997; Bart and Earnst 1999; Rintamaki et al. 2000). These are supra-orbital fleshy ornaments and their size is testosterone dependent (Rintamaki et al. 2000; Mougeot and Redpath 2004). There has been very little consideration of the possible role of plumage colour in male-male interactions or mate choice in either species, particularly the red grouse. Given that moulting in the red grouse occurs prior to territory acquisition and pairing it seems likely that it could play a role in one or both of these.

The melanocortin 1-receptor (MC1R) is responsible for switching melanin pigment synthesis between its two forms. These are pheomelanin, which is red to yellow in colour, and eumelanin, which is dark brown to black in colour. Mutations at *MC1R* that cause increased constitutive activity of the receptor have been found to produce dark coloured fur or feathers in many species of bird and mammal (Takeuchi et al. 1998; Theron et al. 2001; Eizirik et al. 2003; Ling et al. 2003; Nachman et al. 2003; Mundy et al. 2004) and other mutations that reduce the activity of the receptor produce pale or red coloured hair (Valverde et al. 1995; Schiöth et al. 1999; Ritland et

al. 2001). Therefore it seems possible that the divergence in male plumage colour between the red grouse and black grouse could be due to changes at this locus. In the previous chapter I reported an association between the level of sexual plumage dichromatism and the relative rate of amino acid substitution at *MC1R*. This seemed likely to be due sexual selection driving change but an increased rate of change due to decreased constraint and neutral drift could not be ruled out. Comparing the relative numbers of amino acid (nonsynonymous) substitutions and silent (synonymous) substitutions between and within the red grouse and black grouse will provide a further test for positive selection occurring at *MC1R* in relation to male plumage colour (McDonald and Kreitman 1991). The level of intraspecific variation at *MC1R* will also give an indication of whether there is potential for on-going sexual selection at this locus in current populations.

## Methods

### Samples

DNA samples and sequence data for *MC1R* and several additional loci (*AGRP, TYR, DCT* and *TYRP1*) had already been obtained from 2 capercaillie individuals, 3 red grouse and one black grouse (see chapter 2). Samples of blood or soft tissue were obtained from a further 24 black grouse individuals and 19 red grouse individuals. The black grouse samples were from a well-studied population in Central Finland and were collected as part of a previous study (Alatalo et al. 1996). The red grouse samples were from several populations in Scotland and Northern England (see table 4.1 for details).

### Laboratory Methods

DNA was extracted using standard methods. An 811bp segment of the 945bp single coding exon of *MC1R* was amplified using primers MSHR84 (5'-AACCAGAGCAACGCCACA-3') and MSHR85 (5'-CTCCTGGCTCCGGAAGG-3'). PCR was performed in a 50µl total volume containing: 1.0 unit Taq polymerase (Advanced Biotechnologies), 1 x Reaction buffer, 1.5mM $MgCl_2$, 50mM each dNTP, 10nM each primer and 50-200ng DNA. PCR reactions were performed in a DNA Engine (MJ Research), with the following cycling parameters: 94ºC for 2 minutes, 35 x: (94ºC for 30s, 62-65ºC for 45s, 72ºC for 1 minute), 72ºC for 5 mins. PCR products were directly sequenced on both strands using the PCR primers and the two internal primers MSHR73 (5'- GGCGTAGAAGATGGTGATGTAGC-3') and MSHR74 (5'-GTGGACCGCTACATCACCAT-3').

| Species | Sample no. | Location | Estate/area |
|---|---|---|---|
| *T. urogallus* | 6 | Scotland* | - |
| *T. urogallus* | 11 | Scotland* | - |
| *T. tetrix* | 3 | Scotland* | - |
| *T. tetrix* | 1 | Finland | Petäjävesi |
| *T. tetrix* | 2 | Finland | Petäjävesi |
| *T. tetrix* | 4 | Finland | Petäjävesi |
| *T. tetrix* | 5 | Finland | Petäjävesi |
| *T. tetrix* | 6 | Finland | Petäjävesi |
| *T. tetrix* | 7 | Finland | Petäjävesi |
| *T. tetrix* | 8 | Finland | Petäjävesi |
| *T. tetrix* | 9 | Finland | Petäjävesi |
| *T. tetrix* | 10 | Finland | Petäjävesi |
| *T. tetrix* | 11 | Finland | Petäjävesi |
| *T. tetrix* | 12 | Finland | Petäjävesi |
| *T. tetrix* | 13 | Finland | Petäjävesi |
| *T. tetrix* | 14 | Finland | Petäjävesi |
| *T. tetrix* | 15 | Finland | Petäjävesi |
| *T. tetrix* | 16 | Finland | Petäjävesi |
| *T. tetrix* | 17 | Finland | Petäjävesi |
| *T. tetrix* | 18 | Finland | Petäjävesi |
| *T. tetrix* | 19 | Finland | Petäjävesi |
| *T. tetrix* | 20 | Finland | Petäjävesi |
| *T. tetrix* | 21 | Finland | Petäjävesi |
| *T. tetrix* | 22 | Finland | Petäjävesi |
| *T. tetrix* | 23 | Finland | Petäjävesi |
| *T. tetrix* | 24 | Finland | Petäjävesi |
| *T. tetrix* | 25 | Finland | Petäjävesi |
| *L. lagopus* | 47 | County Durham, UK | Raby |
| *L. lagopus* | 48 | County Durham, UK | - |
| *L. lagopus* | 49 | County Durham, UK | Hawkswood |
| *L. lagopus* | 2 | Northumberland, UK | Lilburn |
| *L. lagopus* | 5 | Borders, UK | Hopes & Tollishill |
| *L. lagopus* | 6 | Borders, UK | Burncastle |
| *L. lagopus* | 7 | South Dales, UK | Ramsgill |
| *L. lagopus* | 8 | South Dales, UK | Dallowgill |
| *L. lagopus* | 9 | South Dales, UK | Stean |
| *L. lagopus* | 10 | Aberdeenshire, UK | Forest of Birse |
| *L. lagopus* | 12 | South Dales, UK | Pockstones |
| *L. lagopus* | 13 | Orkney, UK | - |
| *L. lagopus* | 15 | Speyside, UK | Clune |
| *L. lagopus* | 16 | North Yorkshire, UK | Grinton |
| *L. lagopus* | 17 | North Yorkshire, UK | Grinton |
| *L. lagopus* | 18 | North Yorkshire, UK | Grinton |
| *L. lagopus* | 30 | North Yorkshire, UK | Grinton |
| *L. lagopus* | 31 | North Yorkshire, UK | Grinton |
| *L. lagopus* | 32 | North Yorkshire, UK | Grinton |
| *L. lagopus* | 33 | North Yorkshire, UK | Grinton |
| *L. lagopus* | 34 | North Yorkshire, UK | Grinton |
| *L. lagopus* | 35 | North Yorkshire, UK | Grinton |

Table 4.1 Grouse samples used in this study. * Samples obtained from the national Museum of Scotland but their origin is uncertain.

**Analysis**

Haplotypes were reconstructed from genotype data using the program Phase 2.1.1 (Stephens et al. 2001; Stephens and Scheet 2005) with 1500 generations and a burn-in of 100 generations. The algorithm was applied 5 times with different seeds to check for consistency. Haplotype networks were constructed using the program Network with the median joining algorithm and post-processing maximum parsimony calculations. McDonald-Kreitman (MK) tests for adaptive evolution (McDonald and Kreitman 1991) were carried out using a contingency table analysis implemented in the program RxC (Miller 1997). Standard diversity-index calculations and Tajima's D tests of neutrality were performed using Arlequin version 2.000 (Schneider et al. 2002).

## Results

### Intraspecific variation at *MC1R*

There are 13 variable sites in the 811bp of *MC1R* from the sampled red grouse and 10 variable sites among the black grouse. Three of these (all synonymous) are found in both species. It is possible that these are ancestral and have been maintained across successive speciation events, although it seems more likely that they arose independently in the two lineages. In the red grouse 5 of the 13 substitutions were nonsynonymous (38%), whereas only 1 out of 10 (10%) among the black grouse were. However this difference is not significant (p=0.181). Nucleotide diversity indices are similar for both species (table 4.2). In addition Tajima's D values are similar and neither show significant deviations from neutrality.

| | π | S.E. | Tajima's D | |
|---|---|---|---|---|
| Black grouse | 0.0020 | 0.0013 | -0.822 | p=0.22 |
| Red grouse | 0.0027 | 0.0016 | -0.833 | p=0.22 |

Table 4.2 *MC1R* nucleotide diversity (π) and Tajima's statistics for the black grouse and red grouse.

There was no obvious evidence for intraspecific population differentiation at *MC1R*. Both alleles of the Scottish black grouse shared the most common haplotype of the Finnish population (figure 4.1). Similarly the red grouse from Orkney, which might be expected to be the most distinct from the other populations, had two copies of the most common red grouse haplotype. Alleles from the other two more distant populations, Aberdeen and Speyside, belonged to two of the most frequently sampled haplotypes from all the populations.

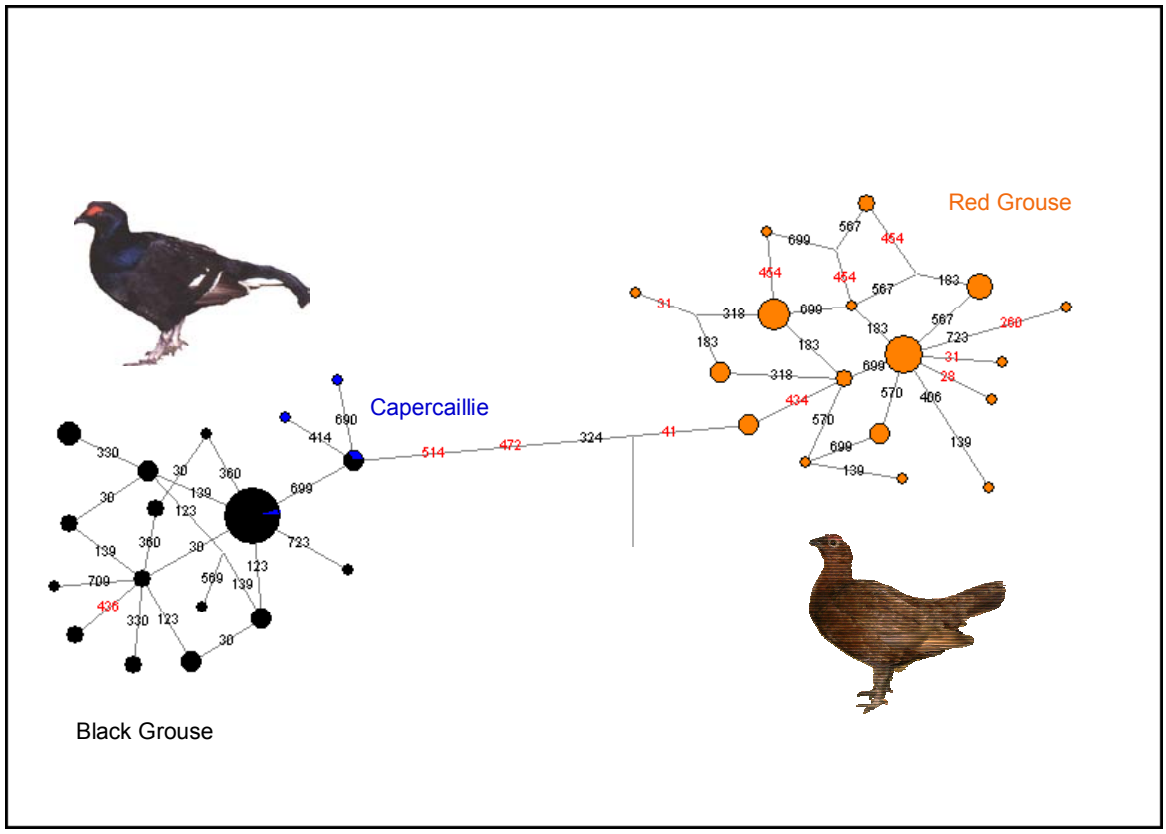



Figure 4.1 Haplotype network for *MC1R* in the black grouse, red grouse and capercaillie. Branch numbers indicate positions of nucleotide substitutions with 1 equal to nucleotide 76 of the complete chicken *MC1R*. Numbers in red indicate nonsynonymous substitutions. Red grouse haplotypes are orange; black grouse, black and capercaillie, blue, with node size proportional to haplotype frequency. Root inferred from previous analyses (chapter 3).

An MK test comparing the fixed differences between the red grouse and black grouse to the number of polymorphic differences within both species indicated that there was not a significant excess of nonsynonymous changes between species (p=0.0754) (table 4.3). When the fixed differences were compared to the polymorphic differences in the black grouse only, there was a significant excess of synonymous substitutions within the black grouse (p=0.0412). This may indicate an adaptive divergence between red grouse and black grouse or could indicate a higher level of purifying selection acting within the black grouse, or a combination of these. However it should be noted that this result is no longer significant when applying a Bonferroni correction for multiple tests (corrected α-level = 0.0167 for 3 tests).

| | | | |
|---|---|---|---|
| All 3 species | | Syn | NS |
| | Fixed | 1 | 3 |
| p=0.075 | Polymorphic | 19 | 6 |
| Black grouse | | Syn | NS |
| | Fixed | 1 | 3 |
| p=0.040 | Polymorphic | 9 | 1 |
| Red grouse | | Syn | NS |
| | Fixed | 1 | 3 |
| P=0.399 | Polymorphic | 8 | 5 |

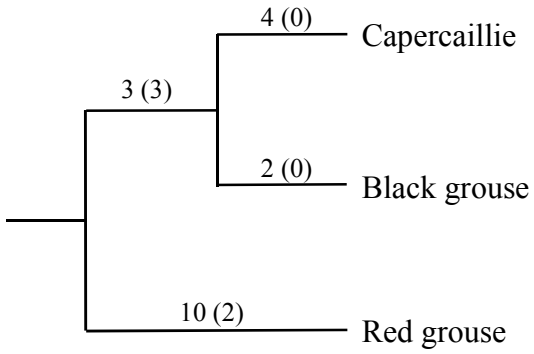
Table 4.3 McDonald-Kreitman tests on red grouse versus black grouse/capercaillie at *MC1R*. For the comparison involving all 3 species, fixed differences between the red grouse and black grouse/capercaillie are compared to polymorphic differences in all 3 species. For the black grouse, fixed differences between the red grouse and black grouse are compared to polymorphic differences within the black grouse only. For the red grouse, fixed differences within the red grouse and black grouse are compared to polymorphic differences within the red grouse only.

**Interspecific divergence at *MC1R***

The red grouse and black grouse *MC1R* sequences are separated by 4 fixed nucleotide differences (figure 4.1). One of these changes is synonymous while the other 3 lead to amino acid replacements. The high relative number of nonsynonymous to synonymous changes may indicate that there has been an adaptive change at *MC1R* between these species. However as reported above this is only significant in the MK test when compared to the polymorphic differences within the black grouse only.

The capercaillie show low divergence from the black grouse at *MC1R* and also share two haplotypes with them. This is consistent with these species having similar dark-coloured plumage and also with them being relatively closely related. A comparison with the *AGRP* locus, which is just over half the length of *MC1R* (422bp), reveals a higher level of divergence with three nucleotide substitutions between the black grouse and capercaillie (figure 4.2). The level of divergence between the red grouse and black grouse is the same with 4 fixed nucleotide substitutions. The ratio of

synonymous to nonsynonymous substitutions however, is far greater with 3 of them being synonymous. A similar pattern is seen at the *TYR* locus, which is a similar length to *MC1R* (772bp): two fixed substitutions are present between the black grouse and capercaillie, with 4 synonymous and 3 nonsynonymous substitutions between the red grouse and the black grouse/capercaillie ancestor.




| | Red grouse to black grouse/capercaillie ancestor | | Black grouse to capercaillie | |
|---|---|---|---|---|
| | syn | NS | syn | NS |
| AGRP | 3 | 1 | 3 | 0 |
| TYR | 4 | 3 | 2 | 0 |
| DCT | 0 | 1 | 0 | 0 |
| TYRP1 | 1 | 0 | 1 | 0 |

Figure 4.2 Divergence between the grouse species at four additional loci. Numbers above the branches indicate the number of substitutions with the number that are nonsynonymous in parentheses. The table to the right gives the details for each of the individual loci for the divergence between the red grouse and black grouse/capercaillie (2 branches combined) and for the divergence between the capercaillie and black grouse (again combining 2 branches).

## Discussion

The results presented here are suggestive of positive selection acting at *MC1R* during the separation of the black grouse and red grouse lineages. These genera are not sister taxa and are likely to have split from their common ancestor 2-5mya (Lucchini et al. 2001; Pereira and Baker 2006). Several recent studies have found evidence that *MC1R* is involved in bringing about colour variation in natural populations (Theron et al. 2001; Eizirik et al. 2003; Nachman et al. 2003; Mundy et al. 2004). Similar to the results presented here, other studies have been strongly indicative yet failed to find conclusive evidence of a role for *MC1R* (Doucet et al. 2004; Rosenblum et al. 2004). These studies have all focused on colour polymorphic populations within species. It is perhaps unsurprising then that when considering such highly diverged species as the grouse it is hard to demonstrate that *MC1R* has been involved in causing changes in plumage colour.

The evidence for an excess of nonsynonymous substitutions between the red grouse and black grouse at *MC1R,* indicating adaptive evolution, is clearly far from conclusive. This was based on an MK test comparing the fixed differences between these species to the polymorphic differences within them. However, a high proportion (4/7) of the nonsynonymous single nucleotide polymorphisms (SNPs) found within the species were present at a low frequency (<5%). It has been suggested that low frequency nonsynonymous SNPs are likely to represent slightly deleterious mutations and that the presence of these in a population can reduce the power of MK tests to detect adaptive evolution (Fay et al. 2001; Eyre-Walker 2006). Further, a higher number of nonsynonymous relative to synonymous substitutions are found at *MC1R* between the red grouse and black grouse than are found at other loci. This is unexpected given the low average dN/dS in the Galliformes at *MC1R* compared to

other loci (chapter 3). And therefore may also be evidence that these changes have been selected to bring about the changes in male plumage colour seen on these lineages. The black grouse and capercaillie have high similarity at *MC1R*, which seems to be higher than the similarity at other loci. This may be due conservation at this locus between these species due to their conserved dark plumage colour. However the number of nucleotide substitutions between species at all loci was small and therefore these results are suggestive at best.

If adaptive evolution has occurred at *MC1R* between the red grouse and black grouse, it seems most likely to have been due to selection acting on male plumage colour as this is highly divergent, whereas female colouration is very similar in these species. Mutations at *MC1R* can have differential effects on the sexes in chickens (Kerje et al. 2003) and therefore it does seem feasible that this locus could play a role in the evolution of plumage colour of males only.

The amino acid substitutions between the red grouse and black grouse are Q34P (corresponding to a nucleotide substitution A41C as numbered on figure 4.1) on the branch leading to the red grouse, and I178V (A472G) and G192S (A514G) on the branch leading to the black grouse. None of these have previously been described has having a phenotypic effect (Majerus and Mundy 2003; Mundy 2005). Q34P represents a more significant amino acid substitution than the other two but is located in the amino-terminal region, which is likely to be less important for receptor function (Garcia-Borron et al. 2005). On the other hand the substitutions on the black grouse lineage are relatively minor amino acid changes but occur at the plasma membrane-extracellular medium interface between extracellular loop 2 and transmembrane regions 4 and 5. These interface regions are where ligand binding occurs and the length of extracellular loop 2 is thought to be related to the level of constitutive

activity (Garcia-Borron et al. 2005). Therefore it is possible that even minor changes in these positions could have an effect on receptor activity.

One of the amino acid polymorphisms found in the red grouse (R107H) is at the homologous position to one of the four that was found in dark rock pocket mice (R109W) (Nachman et al. 2003). In the rock pocket mice all four substitutions were found in complete linkage disequilibrium in all dark mice and so it is hard to say what the phenotypic effect of any one of them would be. I have no information on the phenotypes of the birds in this study and therefore do not know if the bird possessing this substitution showed any difference in feather colour. A colour polymorphism in the down of newly hatched red grouse chicks has been described (Henderson 1977) and there is also known to be some variation in adult pigmentation (Madge and McGowan 2002). Although, the variation in adult plumage colour seems to be fairly complex and unlikely to be due to a single locus, this does not rule out a role for *MC1R* in causing some of the variation. In contrast black grouse seem to be much less variable in plumage colour, but distinct geographical races across Europe do exist. Variation in male plumage is mostly in the colour of the sheen on their black plumage, while females vary from pale to dark brown (Madge and McGowan 2002).

Levels of *MC1R* nucleotide diversity are similar between the red grouse and black grouse. The slightly higher diversity in the red grouse may be due to the wider sampling of populations in this species. The value found for the black grouse is just over half that found in several island populations of the bananaquit (*Coereba flaveola*) ($\pi \approx 0.0038$), a small passerine bird (Theron et al. 2001). Similar nucleotide diversity values have also been reported in two further passerine species, the willow warbler (*Phylloscopus trochilus*) ($\pi = 0.0038$) and dusky warbler (*P. fuscatus*) ($\pi = 0.0025$) (Bensch et al. 2006). A higher value was reported in the chiffchaff (*P. collybita*) ($\pi =$

0.0137), although this may have been partly due to sampling of a small number of individuals across divergent subspecies. Nucleotide diversity is expected to be lower in the grouse than these passerines given the larger body size and smaller population sizes of the grouse. The reasonably high nucleotide diversities in the grouse therefore suggest that *MC1R* has not recently been under strong directional selection in either of the grouse species. This is also backed up by the lack of deviation from neutrality in the Tajima's D tests. The lack of on-going directional selection at *MC1R* in either of the species is consistent with the field data suggesting that plumage ornaments are not currently important in determining mate choice (Alatalo et al. 1991; Rintamaki et al. 1997) although little attention has been paid to plumage colour per se.

There is, however, some evidence for ongoing purifying selection at *MC1R* in the black grouse. This species shows an excess of synonymous substitutions that is not found in the red grouse suggesting a higher level of purifying selection. This supports the conclusion that the increased dN/dS at *MC1R* associated with high dichromatism, reported in chapter 3, is due to positive selection not reduced purifying selection. If *MC1R* was important in generating the male plumage colour of the black grouse it is easy to imagine that the strong sexual selection pressure operating on the male black grouse would rapidly remove any new mutations arising at this locus that had an effect on plumage colour. Such mutations would presumably occur at low frequency and so it is unlikely that they would be observed or found to have a significant effect in studies of mate choice. It therefore seems possible that *MC1R* was under evolving under positive sexual selection pressure at a time after the split of the red grouse and black grouse. It then became fixed in the ancestor of the black grouse and capercaillie and since that time has been maintained in the black grouse under strong purifying selection. In order to test this hypothesis further it would be

interesting to obtain *MC1R* sequence from more grouse species to identify the exact branches on which the amino acid substitutions occurred. In addition, sampling of other loci at the population level would permit further testing of the possible departure from neutrality at *MC1R* in the divergence of the black and red grouse (Hudson et al. 1987). An investigation of intraspecific variation at other pigmentation loci in the black and red grouse would also be interesting.

# Chapter 5

# The molecular genetics of plumage colour mutants in Japanese quail (*Coturnix japonica*) and sexual dichromatism in the common pheasant (*Phasianus colchicus*)


## Abstract

**The genetic basis of avian plumage colour is poorly understood especially compared to that of mammalian hair colour. Here I use a candidate gene approach to investigate the genetic basis of three quail pigmentation variants: extended brown, roux and yellow. Extended brown is found to be associated with a single nucleotide substitution at *MC1R* leading to a Glu92Lys amino acid substitution. The same substitution has also been found to be associated with dark phenotypes in mice, chickens and bananaquits. Roux is associated with a single nucleotide substitution at *TYRP1* that causes a Phe282Ser substitution. The yellow variant is found to be due to a regulatory change at the *ASIP* locus, similar to that found in the lethal yellow, mouse mutation. This is the first time that a transcribed *ASIP* gene has been described from avian skin together with evidence for alternative splicing leading to a ventral specific *ASIP* transcript, as is found in pale bellied mice. Finally a comparison of candidate gene expression in the skin of male and female common pheasants revealed significantly lower expression of the Z-linked gene *TYRP1* in males.**

## Introduction

Avian plumage colour has long been of interest to behavioural biologists because of its role in signalling and mate choice (Andersson 1994; Irwin 1994; Owens and Hartley 1998; Figuerola and Green 2000; Dunn et al. 2001). However our understanding of the pigmentation genetics of birds has lagged behind that of mammals. This is perhaps partly because of the increased complexity of feather compared to hair colouration: hair colour is produced entirely by melanin pigments, whereas feathers can also have fine structures that produce colour by scattering light (structural colours) and may contain carotenoid pigments (although it is unlikely that gallinaceous feathers contain these). In addition feathers can be patterned in two dimensions, while hairs are essentially one-dimensional. The mechanisms of melanin synthesis and deposition in birds and mammals are considered to be very similar despite the fact that they evolved independently from the pigmentation system found in reptiles, which appears to be quite different (Rosenblum et al. 2004). Several pigmentation genes known in mammals have now been cloned and sequenced in birds, primarily chickens (*Gallus gallus*) (Mochii et al. 1992; Takeuchi et al. 1996; April et al. 1998a; April et al. 1998b; Klovins and Schiöth 2005). The classical genetics of plumage colour in chickens has been extensively studied by chicken breeders and revealed in excess of 40 loci capable of altering melanic pigmentation (Smyth 1990). However, a link between these and the molecular genetics has only been demonstrated in a handful of cases.

Perhaps the best characterised of these is the melanocortin 1-receptor gene (*MC1R*), which is known in mammals to be situated in the melanocyte membrane and involved in switching pigment synthesis from pheomelanin to eumelanin (Robbins et al. 1993). Several functional variants at this locus (the *E* locus) are now known in

chickens, with dominant mutations producing a darker phenotype (*E, $E^R$*) and recessive mutations producing a paler phenotype ($e^b$, $e^y$) (Takeuchi et al. 1998; Kerje et al. 2003). The *E* allele contains a glutamate to lysine substitution at amino acid position 92 and this leads to the production of a constitutively active receptor (Ling et al. 2003). This mutation is also found in a naturally occurring colour polymorphic bird species, the bananaquit (Theron et al. 2001), and other mutations at this locus are associated with colour polymorphisms in two further wild bird species (Mundy et al. 2004).

The molecular basis of two further chicken pigmentation loci have been demonstrated. The first of these is the *C* locus. This has been identified as the tyrosinase gene (*TYR*), which codes for a key enzyme in melanogenesis. Two different mutations at this locus have been found to be responsible for the autosomal albino ($c^a$) (Tobita-Teramoto et al. 2000) and the recessive white (*c*) phenotypes (Chang et al. 2006). The second is the *I* locus, which encodes the PMEL17 protein. This is a melanosomal membrane protein crucial for the normal development of eumelanosomes. The dominant white (*I*), smoky ($I^s$) and dun ($I^d$) phenotypes were all found to be due to mutations at *PMEL17* (Kerje et al. 2004).

Japanese quail have been less intensively studied than chicken. Some quail plumage colour mutants have obvious chicken homologues and this can be tested directly using chicken/quail hybridisation experiments (Minvielle et al. 2002). However there are other quail loci that do not have obvious homologues in chickens (Cheng and Kimura 1990). Therefore investigating quail pigmentation genetics can extend our understanding of the avian pigmentation system in new directions as well as providing a source of comparison for the chicken system. To date one quail pigmentation locus has been characterised at the molecular level. This is the silver (*B*)

locus, which was found to encode MITF (Mochii et al. 1998). This is not homologous to the chicken silver locus (*S*), which is sex-linked and primarily involved in pheomelanin expression. MITF is a transcription factor that plays a central role in the signalling pathways controlling the survival, proliferation and differentiation of melanocytes, including regulation of *TYR, TYRP1, DCT, MC1R* and *PMEL17* (Vance and Goding 2004).

Perhaps the most studied of the Japanese quail pigmentation mutations is black at hatch (*Bh*), controlled by an autosomal dominant gene with homozygous lethality. This appears to be involved in pigmentation pattern formation (Niwa et al. 2002), is expressed in the melanocytes (Satoh et al. 1997) and has been mapped to quail chromosome 1 (Niwa et al. 2003; Miwa et al. 2006). No likely candidates for this locus are known in mammals and similar mutations are not found in chickens. Similarly the quail panda mutation (*s*), which produces overall white plumage with spots of wild-type colour, has been mapped to a 4.5Mb region of chromosome 4. In this case a candidate for this locus has been proposed: *EDNRB2*, a paralogue of mammalian *EDNRB*, which is responsible for spot colour in mammals (Miwa et al. 2006).

In this study I investigate the molecular genetic basis of three further quail pigmentation mutations. The first of these, extended brown (*E*) seems a likely homologue of the chicken *E* locus and therefore *MC1R* will be investigated as a candidate for this locus. However *E/E* quails are paler than *E/E* chickens, showing all-over brown colouration rather than all-over black colouration (figure 5.1a). The quail *E* allele is also incompletely dominant with $E/e^{+}$ individuals having intermediate plumage colour and showing signs of the pale ventral colouration of wild-type individuals. Secondly I study the roux mutation. This is a recessive sex-linked

mutation and has been found to be allelic to the sex-linked brown mutation (Minvielle et al. 2000) and so has been given the symbol $br^r$. These mutations both result in a dilution of eumelanin pigmentation with brown being slightly darker and dominant to roux (figure 5.1b). Sex linked mutations with these characteristics have not been described in chickens. These phenotypes are similar to those seen in *brown* mice, having a mutation in the tyrosinase-related protein-1 gene (*TYRP1*) (Zdarsky et al. 1990). TYRP1 is an enzyme in the tyrosinase gene family but, unlike tyrosinase, is thought to only be involved in eumelanin and not pheomelanin synthesis. Chicken *TYRP1* is located on the Z chromosome and so is very likely to also be sex linked in the Japanese quail. Therefore *TYRP1* seems a good candidate gene for the *BR* locus in quail.

The third quail variant that I consider is Yellow (*Y*) (figure 5.1c). This is an autosomal dominant mutation with homozygous (*Y/Y*) lethality. The effects of this mutation on pigmentation are similar to the mouse lethal yellow ($A^y$) mutation, which is due to a 170Kb deletion upstream of the mouse agouti signalling peptide gene (*ASIP*). This deletes the coding region of the *Raly* gene and puts *ASIP* under the control of the *Raly* promoter (Michaud et al. 1994). ASIP is an endogenous antagonist of MC1R and so expression decreases eumelanin and increases pheomelanin production. $A^y$ causes ubiquitous expression of *ASIP*, which produces the yellow coat colour and also mimics the effect of the agouti-related protein (AGRP) on MC3R and MC4R in the brain causing overeating and obesity.

Until recently it was thought that birds did not possess a functional *agouti* gene (Boswell and Takeuchi 2005). This was partly due to failed attempts to clone *ASIP* in chicken and the finding of peripheral expression of *AGRP*, which it was thought may take the role of *ASIP* in pigmentation (Takeuchi et al. 2000). There is also a lack of

obvious agouti-like mutations in chickens, compared to more than 30 agouti alleles that have been described in mice (Siracusa 1994), which may indicate that an antagonist of *MC1R* is absent or highly constrained in birds. However, an *ASIP*-like sequence has now been identified on chicken chromosome 20 (Klovins and Schiöth 2005). Evidence that *Y* may be a mutation of avian *ASIP* came from a mapping study that mapped *Y* to the quail chromosome homologous to chicken chromosome 20 (Miwa et al. 2006). In addition crossing experiments between extended brown and yellow quails indicate that *E* is epistatic to *Y* (Minvielle, personal communication), which would be consistent with these loci being *MC1R* and *ASIP* respectively, given that increased *ASIP* expression would have no effect on a constitutively active MC1R. If *Y* is a mutation of *ASIP* it will be the first evidence that this locus is functional and plays a role in pigmentation in birds.

Another reason that quails are interesting in regard to the *ASIP* locus is that they possess a pale ventral and dark dorsal pigmentation pattern (figure 5.1d). In pale-bellied ($A^w$ or $a^t$) mice, which is likely to be the ancestral phenotype (Chen et al. 1996), this type of patterning is produced by ventral specific expression of *ASIP* (Vrieling et al. 1994). Four different *ASIP* transcripts are produced in mice, produced by differential transcription of three different non-coding exons (1A, 1B and 1C). One of these (1A) is expressed only in the ventral skin of $A^w$ or $a^t$ mice. Investigation of natural expression patterns of ASIP in Japanese quail could therefore tell us if this locus is also involved in dorsoventral pigmentation patterning in quails and if different regulatory transcripts are involved.

Finally I use some of these candidate genes that have been identified in quail to study the production of sexually dichromatic plumage in the common pheasant (*Phasianus colchicus*). Experiments involving dulling of male plumage in this species

suggest that plumage colour is used in male-male competition (Mateos and Carranza 1997). Males of this species are overall darker than females and also show a strong reddish colour over most of the body feathers and a dark almost black head with a structural blue/green sheen (figure 5.1e,f). The red colour of the body feathers is presumably due to pheomelanin although the presence of a slight red sheen suggests that it is also enhanced by structural colour.


Figure 5.1 Plumage colour phenotypes in this study. Japanese quail plumage colour mutants: **a**) extended brown heterozygote (*E/e*$^{+}$) (left) and homozygote (*E/E*) (right) phenotypes; **b**) the brown locus with wild-type (left), brown (centre) and roux (right) phenotypes; **c**) yellow phenotype; **d**) wild-type male (left) and female (right). Common pheasant plumage colour: **e**) adult male; **f**) adult female; **g**) 8-week-old male (sampled); **h**) 8-week-old female (sampled). Quail photographs courtesy of F. Minvielle; male pheasant photograph courtesy of J. Corder.

## Methods

### Extended brown (*E*) and *MC1R* in Japanese Quail

Initially a set of DNA samples from 2 wild type and 2 extended brown Japanese quail from France and 1 extended brown individual of a darker phenotype from Gifu University, Japan were obtained. Following this soft tissue samples were obtained from 24 individuals from 2 families segregating for extended brown *(E)* reared at the Unité Expérimentale de Génétique Avicole, INRA, Nouzilly, France; 8 homozygous extended brown (*E/E*), 8 heterozygous $E/e^+$ and 8 homozygous wild-type ($e^+/e^+$).

Genomic DNA was extracted using standard methods. For the initial 5 samples an 859bp segment of the 945bp single coding exon of *MC1R* was amplified using primers MSHR72 and MSHR78 (see table 5.1 for primer sequences). For the following samples a 642bp fragment encompassing all variable sites found in the initial screen was amplified using primers MSHR82 and MSHR83. PCR reactions were performed in a 50µl total reaction containing: 1.0 unit Taq polymerase (Advanced Biotechnologies), 1 x Reaction buffer, 1.5mM $MgCl_2$, 50mM each dNTP, 10nM each primer and 50-200ng DNA. PCR reactions were performed in a DNA Engine (MJ Research), with the following cycling parameters: 94ºC for 2 minutes, 35 x: (94ºC for 30s, 65-68ºC for 45s, 72ºC for 1 minute), 72ºC for 5 mins. PCR products were directly sequenced on both strands using the PCR primers and the two internal primers MSHR73 and MSHR74.

| Primer | Sequence (5'-3') | Locus | Direction |
|---|---|---|---|
| ACT1F | TGCGTGACATCAAGGAGAAG | *β-actin* | Forward |
| ACT1R | CAGGTCCTTACGGATGTCCA | *β-actin* | Reverse |
| ASIPF1 | AAAAGGAAGAACCTTTTCCTAAGC | *ASIP* | Forward |
| ASIPF2 | TCATTTTCATGACAGTGGGATT | *ASIP* | Forward |
| ASIPF5 | CCAACAATGAAAAGGAAGAACC | *ASIP* | Forward |
| ASIPF6 | TCTCCTCGGCTATATGGCT | *ASIP* | Forward |
| ASIPF7 | GAAGCAGGCAGTCTTCTTGG | *ASIP* | Forward |
| ASIPF8 | CCAGCATTTTCATATTTTCTGGA | *ASIP* | Forward |
| ASIPF9 | GCTGCCAGATCTCACCTGAT | *ASIP* | Forward |
| ASIPF11 | TCTTCCTCCTCCTCCTCCTC | *ASIP* | Forward |
| ASIPR1 | CATTTGCACAACGCACAGTA | *ASIP* | Reverse |
| ASIPR5 | GATTTGGTTTAACACTTTGGGTTT | *ASIP* | Reverse |
| ASIPR6 | TTTGGGGGTGTCTTCAGTTC | *ASIP* | Reverse |
| ASIPR7 | GTTCATTTTGTTGTTCTTTGACAG | *ASIP* | Reverse |
| ASIPR9 | AAAACGTGCAGCAAAACC | *ASIP* | Reverse |
| ASIPR10 | TCTGGGAGGTTCATTTTGTTG | *ASIP* | Reverse |
| DCTF2 | TGCTGGCTATAACTGTGGTGA | *DCT* | Forward |
| DCTR2 | CTTTCCAGCAACAGCAAATG | *DCT* | Reverse |
| GAPDHF1 | CTAAGGCTGTGGGGAAGGTCA | *GAPDH* | Forward |
| MSHR72 | ATGCCAGTGAGGGCAACCA | *MC1R* | Forward |
| MSHR73 | GGCGTAGAAGATGGTGATGTAGC | *MC1R* | Forward |
| MSHR74 | GTGGACCGCTACATCACCAT | *MC1R* | Reverse |
| MSHR78 | CAGGAGCACAGCACCACCTC | *MC1R* | Reverse |
| MSHR82 | GACATCCCCAATGAGCTGTT | *MC1R* | Forward |
| MSHR83 | AGGTGACGATGAGGATGAGG | *MC1R* | Reverse |
| TP1e1F3 | CTCAGTTCCCTCGCCAGT | *TYRP1* | Forward |
| TP1e1R1 | GATTTGCTGGCTACAGGTAGGTC | *TYRP1* | Reverse |
| TP1e2R1 | GCATGTCCCTTTCAAGTTGC | *TYRP1* | Reverse |
| TP1F4 | CAGCTCTGCTGAACCTGTTG | *TYRP1* | Forward |
| TP1F5 | TCAGAAGGAATCTTTTGGATCTT | *TYRP1* | Forward |
| TP1F6 | AAATACGACCCGGCAGTTC | *TYRP1* | Forward |
| TP1R5 | ATTGGTTTTAGTCACAAGCAAAAA | *TYRP1* | Forward |
| TP1R6 | GCCACTCATCAAAAACAGCA | *TYRP1* | Reverse |
| SJ2 | CATCAAAGGTGGAGGAATGGC | *GAPDH* | Reverse |
| SJ5 | CCGGATATGCGCTGGAATAAG | *MITF* | Forward |
| SJ6 | CAAGCATGATCAGTGTCCTCC | *MITF* | Reverse |

Table 5.1 Primers used in this study for PCR and sequencing. See text for details of PCR conditions and amplified fragment lengths.

**Roux *($br^r$)* and *TYRP1* in Japanese Quail**

Dorsal skin samples were obtained from 12 male individuals, 6 with a roux phenotype (*$br^r$/ $br^r$)* and 6 with a wild-type phenotype (*$br^r$ /$BR^+$*), from a single family segregating for the roux mutation. Feathers were plucked from the region of skin that would be sampled 11 days prior to sampling, to stimulate feather growth. Skin samples were taken by dissecting a piece of skin (approx. 4cm$^2$), which was snap frozen in liquid nitrogen and stored at –80ºC.

Total RNA was extracted from the skin samples using the RNeasy mini-kit (Qiagen). RNA concentration, purity and integrity (RIN values) were checked using a BioAnalyser (Agilent). RNA was stored at –80ºC until needed. cDNA syntheses were performed in a 20µl volume with 1-3µg total RNA and 150ng/µl N6 primer using Superscript RT II (Invitrogen) and following the manufacturers instructions. The entire 1611bp coding region of *TYRP1* and 13bp of flanking sequence were amplified using primers TP1F4 and TP1R5, designed based on quail *TYRP1* mRNA sequence with accession AB005228. PCR reactions were performed as described above using 4µl of product from the cDNA reactions, with the following cycling parameters: 94ºC for 2 minutes, 35 x: (94ºC for 30s, 60ºC for 45s, 72ºC for 1 minute), 72ºC for 5 mins. Direct sequencing was performed using the PCR primers and 5 internal primers: TP1F5, TP1F6, TP1R6, TP1e2R1 and TP1e1R1. Quantitative RT-PCR for *TYRP1* was performed as described below using primers TP1e1F3 and TP1e2R1 and for *MITF* using primers SJ5 and SJ6.

**Dorso-ventral pigmentation patterning and *ASIP* in Japanese quail**

Skin samples were obtained from the back and belly of 6 male wild-type individuals. Feathers were cut back prior to the skin being dissected and frozen as described above. RNA was extracted and cDNA syntheses performed as described above. 361-

384bp of the 393bp coding region of *ASIP* was amplified using primers ASIPF2 or ASIPF5 and ASIPR5. 5' non-coding regions identified from EST data in the chicken genome were amplified using forward primers (ASIPF6, ASIPF7, ASIPF8) designed to the predicted non-coding regions and a reverse primer (ASIPR6) within the coding region. PCR reactions were performed as described above with the following cycling parameters: 94ºC for 2 minutes, 40-45x: (94ºC for 30s, 57-60ºC for 45s, 72ºC for 1 minute), 72ºC for 5 mins. PCR products were directly sequenced using the PCR primers. Quantitative RT-PCRs for the coding region of *ASIP* were performed using primers ASIPF1 or ASIPF9 and ASIPR1 as described below.

**Yellow *(Y)* and *ASIP* in Japanese quail**

Dorsal skin samples were obtained from 6 male dominant yellow individuals and 6 male wild-type individuals from 2 segregating families (24 individuals in total). One of these families was plucked prior to sampling, as described above, while the other was not. The skin was dissected and frozen as described above. RNA was extracted and cDNA syntheses performed as described above. The coding and known 5' non-coding regions of *ASIP* were amplified as above. PCR products were directly sequenced using the PCR primers.

To identify the 5' non-coding regions associated with the *Y* allele 5' RACE (rapid amplification of cDNA ends) was performed on a dominant yellow individual and a wild-type sibling as a control. This involves adding to the 5' end, a known adaptor sequence for which a complimentary forward primer is available. This allows PCR amplification of a fragment from the 5' end to a gene-specific primer. The Invitrogen 5' RACE System, version 2.0 was used according to the manufacturers instructions. The gene-specific primer used for cDNA synthesis was ASIPR1. The second gene-specific primer, which was used for the first round of PCR amplification

was ASIPR6. Further gene-specific primers, ASIPR7 and ASIPR9, designed to bind only to the *Y* allele, were then used to perform secondary PCRs. Products were then directly sequenced using the PCR primers and the novel region of non-coding sequence compared to the chicken genome using a BLAST search. The presence of this transcript in only the yellow samples was then confirmed by a PCR using the original cDNA samples from the yellow and non-yellow individuals with primers ASIPF11 and ASIPR10.

Quantitative RT-PCRs for the coding region of *ASIP* were performed in the yellow versus wild-type samples in the same way as wild-type dorsal versus ventral samples.

**Sex differences in gene expression in Common Pheasant Skin**

Skin samples were obtained from the shoulder regions of 8 male and 8 female, 8-week-old, common pheasants from Spindle Wood Game Farm, Suffolk, UK. These birds were beginning to moult into adult sexually dichromatic plumage (figure 5.1g,h). Feathers were cut back prior to the skin being dissected and frozen as described above.

Quantitative RT-PCRs for *TYRP1*, *MITF* and the coding region of *ASIP* were carried out using the primers mentioned above and for *DCT* using primers DCTF2 and DCTR2. These loci were chosen because of their known effects on pigmentation and because RT-PCR protocols had been established for them. A wider screen of more loci is planned.

**Quantitative RT-PCR**

Only samples with RIN (RNA integrity number) values of 8 or above were used for quantitative RT-PCR. cDNA syntheses were carried out as described above.

Quantitative RT-PCR reactions were performed in a 25µl total reaction containing: 1x SYBR Green master mix (Qiagen), 10nM each primer and 1-2.5µl of product from the cDNA reactions. Reactions were performed in an Opticon 2 DNA engine (MJ Research), with the following cycling parameters: 95ºC for 15 minutes, 40-55 x: (94ºC for 15s, 55-68ºC for 30s, 72ºC for 30s), 72ºC for 10 mins. Melting curves were generated between 55 and 90ºC with readings taken every 0.2ºC for each of the products to check that a single product was generated. At least one product from each set of primers was also run on a 1% agarose gel to check that a single product of the expected size was produced and in most cases the identity of the product was confirmed by direct sequencing. In addition to the target loci described above quantitative RT-PCR was also performed for 2 housekeeping control loci *β-actin* and *GAPDH* with primers ACTF1, ACTR1 and GAPDHF1, SJ2 respectively. Amplified fragments for each of the loci were between 247-597bp and always spanned at least one intron to ensure that genomic DNA contamination could be identified.

$C_t$ values were defined as the point at which fluorescence crossed a threshold ($R_{Ct}$) of 10x SD of the background fluorescence. Amplification efficiencies (E) were calculated either using a dilution series of clean PCR product or using the DART-PCR method (Peirson et al. 2003). For all comparisons DART-PCR was used to check that amplification efficiencies did not differ significantly between groups. Starting fluorescence, which is proportional to the starting template quantity, was calculated as $R_0 = R_{Ct} (1+E)^{-Ct}$. Normalised values were then obtained by dividing $R_0$ values for the target loci by $R_0$ values for *β-actin* or *GAPDH.* All results were taken as averages of triplicate PCR reactions and PCRs on target and control loci were always performed using product from the same cDNA synthesis reaction. Relative expression levels were calculated by assigning a value of 1 to the average $R_0$ of all individuals in the

control set (wild-type individuals in the mutant/wild-type comparisons, dorsal samples in the ventral/dorsal comparisons and females in the male/female comparisons). Statistical significance was assessed using unrelated samples 2-tailed t-tests assuming unequal variance.

## Results

### Extended brown (*E*) and *MC1R* in Japanese Quail

The initial screen of 2 wild-type and 3 extended brown individuals revealed 4 variable sites in *MC1R*, 2 of which were non-synonymous. One of these – a G to A substitution leading to a Glu92Lys mutation – appeared to be associated with plumage phenotype. Co-segregation of the Glu92Lys mutation with the extended brown phenotype was confirmed in the two families segregating for *extended brown.* There was perfect and significant association between the presence of a Lys92 allele and the *E* allele ($P < 0.001$, Fisher's exact test). Perfect associations with phenotype were not found for any of the other variable sites (table 5.2).

| | Amino acid | I58V | A59 | E92K | F289 | Accession |
|---|---|---|---|---|---|---|
| Genotype | Nucleotide | 172 | 177 | 274 | 627 | no. |
| $e^+/e^+$ 1 | | G/A | T | G | T/C | |
| $e^+/e^+$ 2 | | G | T | G | T | DQ395091 |
| *E/E* 1 | | A | T | A | C | DQ395089 |
| *E/E* 2 | | A | T | A | C | |
| *E/E* 3 * | | G | C | A | C | DQ395090 |
| *E/E* family 1 (n=3) | | G | C | A | C | |
| *E/E* family 2 (n=5) | | G | C | A | C | |
| $E/e^+$ family 1 (n=3) | | G | T/C | G/A | T/C | |
| $E/e^+$ family 2 (n=5) | | G | T/C | G/A | T/C | |
| $e^+/e^+$ family 1 (n=3) | | G | T | G | T | |
| $e^+/e^+$ family 2 (n=5) | | G/A | T | G | T/C | |

Table 5.2 *MC1R* genotypes of the quails in this study at all variable nucleotide sites. * This individual was of a darker phenotype from Grifu, Japan. No variation was found between individuals within families for each of the *E* phenotypes.

The Glu92Lys mutation in *MC1R* has been previously documented in association with melanism in mice, chickens and bananaquits (Robbins et al. 1993; Takeuchi et al. 1998; Theron et al. 2001). It is therefore likely that this is the causative mutation of extended brown in quails. There were no consistent differences found in the sampled region of *MC1R* between the darker extended brown individual from Gifu, Japan and those of a paler phenotype from the French population.

**Roux *($br^r$)* and *TYRP1* in Japanese Quail**

A single variable nucleotide site was found at *TYRP1* in the family segregating for the roux mutation. This T to C substitution at nucleotide position 845 is nonsynonymous, leading to a phenylalanine to serine mutation at amino acid 282. There was a perfect and significant association between the homozygous presence of the Ser282 allele and the roux phenotype (p=0.001, Fisher's exact test); all of the roux individuals were homozygous for the Ser282 allele and the wild-type siblings were heterozygous Phe282/Ser282 (table 5.3), which is consistent with the recessive nature of this mutation. The published wild-type Japanese quail *TYRP1* sequence (Mochii et al. 1998) was homozygous for the Phe282 allele. There were six additional variable nucleotide positions between this individual and the family sampled here. Five of these were synonymous and one led to an alanine to valine amino acid substitution at position 21.

| Amino acid | A21V | F243 | F282S | V318 | I421 | C498 | N505 |
|---|---|---|---|---|---|---|---|
| Nucleotide | 62 | 729 | 845 | 954 | 1263 | 1494 | 1515 |
| $BR^{+}$ * | C | C | T | T | C | T | T |
| $BR^{+}/br^{r}$ (n=6) | T | T | T/C | C | T | C | C |
| $br^{r}/br^{r}$ (n=6) | T | T | C | C | T | C | C |

Table 5.3 *TYRP1* genotypes of the quails in this study and previously published quail *TYRP1* sequence * (accession no. AB005228) showing all variable nucleotide positions. No variation was found between individuals within the sampled family for each of the *BR* phenotypes.

Comparison of *TYRP1* expression revealed a slightly lower but non-significant difference (p= 0.29, t-test) in the roux compared to the wild-type samples. A very similar reduction was seen in *MITF* expression in the roux samples (figure 5.2).

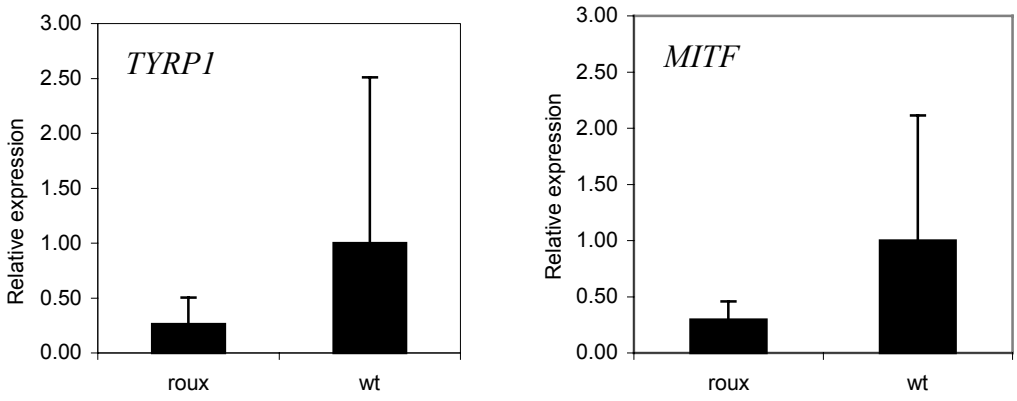



Figure 5.2 Mean relative expression of *TYRP1* and *MITF* in the skin of the roux quails (n=6) compared to the wild-type quails (wt, n=6). Error bars show standard deviations.

**Dorso-ventral pigmentation patterning and *ASIP* in Japanese quail**

An amplicon of the predicted length was obtained by RT-PCR of *ASIP* from quail skin. The sequenced fragment matched the chicken *ASIP* coding sequence predicted by Klovins and Schiöth (2005) confirming the predicted coding exons and suggesting that this sequence does indeed encode an avian *ASIP* gene that is expressed in the skin. In addition to the predicted start codon, which gives the greatest homology to other *ASIP*s, an alternative in-frame start codon was also found 27bp upstream (figure 5.4). This end of the ASIP protein acts as a signal peptide mediating secretion. To investigate what affect the alternative start codon would have on peptide secretion, if it was functional, this region of sequence with or without the extra 9 amino acids was analysed in the program signalP 3.0 (Nielsen et al. 1997; Bendtsen et al. 2004). This revealed that the predicted start codon produced a better signal peptide than the alternative upstream start codon (S=0.75 and S=0.64 respectively). It therefore seems likely that the predicted start codon is the functional one.

Comparison of *ASIP* expression revealed significantly higher expression in the ventral than dorsal samples (figure 5.3) (p=0.036, Wilcoxon's signed ranks test). Very similar results were obtained for expression normalised to *β-actin* or *GAPDH* and for 2 different sets of primers designed to the *ASIP* coding region.

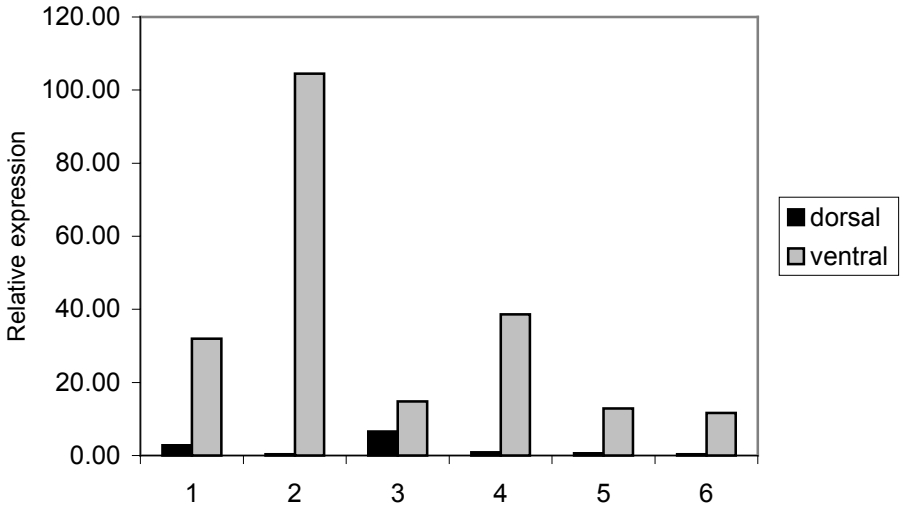



Figure 5.3 Relative expression (normalised to *β-actin*) of coding *ASIP* transcripts in ventral compared to dorsal skin samples from 6 wild-type Japanese quail.

PCR using primers designed to three potential 5' non-coding exons all produced amplicons. Two of these, which I will refer to as exon 1a and exon 1c, were amplified from both dorsal and ventral samples. Sequencing of these amplicons (figure 5.4) revealed that beyond the primer sequence exon 1a was identical to 1c and therefore 1a may not represent a unique transcript. Exon 1b was only amplified from the ventral samples (figure 5.4) and sequencing revealed that this was indeed a different transcript.

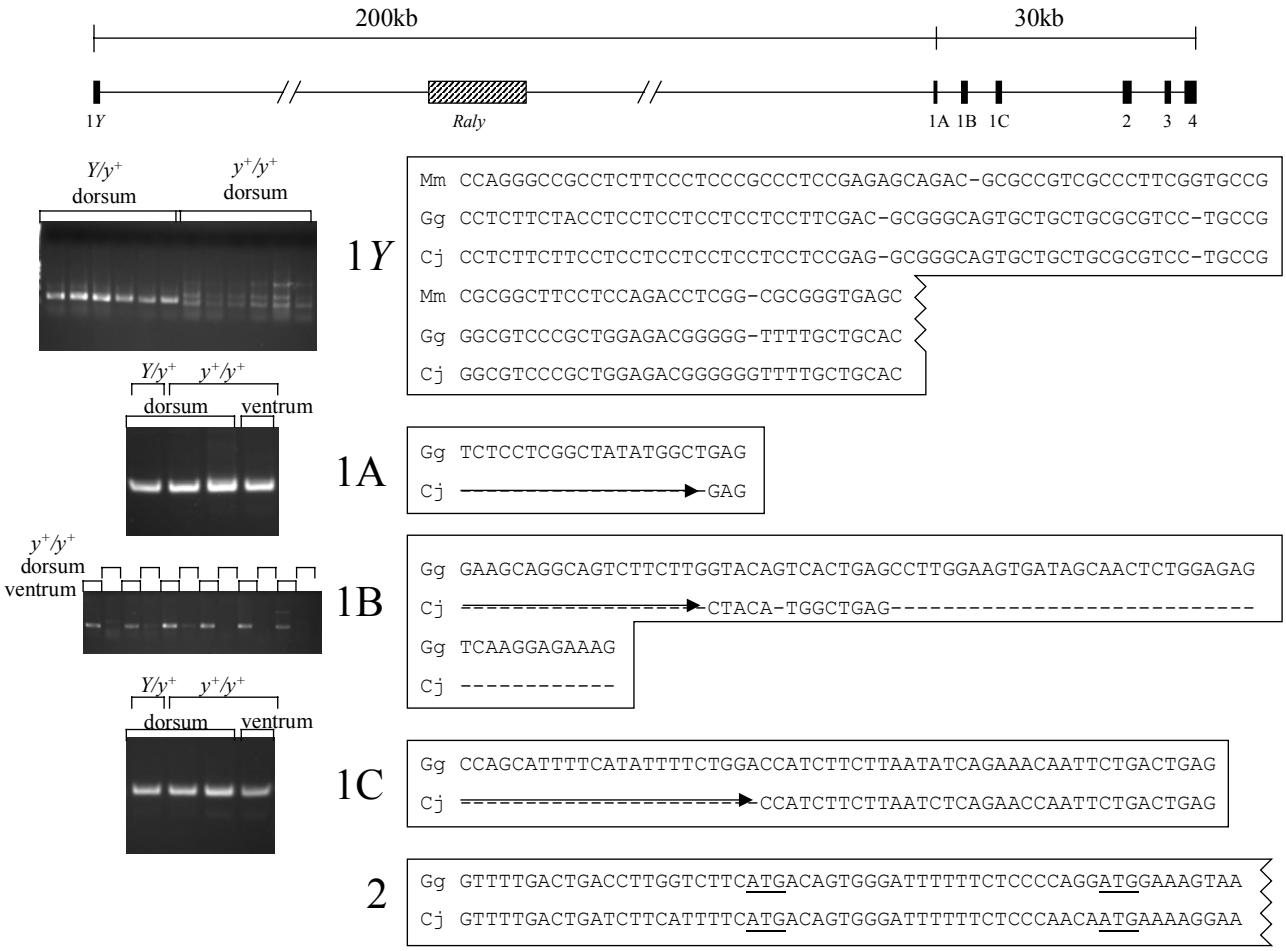



Figure 5.4 Positions, nucleotide sequences and expression patterns of the 3 avian *ASIP* non-coding exons and the 5' non-coding sequence associated with the *Y* allele. The top panel shows the positions of the *ASIP* exons and sequenced region associated with the *ASIP Y* allele (1*Y*) in relation to the *Raly* gene on chicken chromosome 20. The right hand panels show the sequences of each of the 5' non-coding exons with the chicken (Gg) EST sequences (accession no. BU206868/BU337545) from which the primers were designed (marked with arrows) aligned to the quail (Cj) sequences. Within the alignments, missing nuleotides are indicated by dashes. For exon 1B the putative splice site in the chicken is beyond that identified in the quail. The 5' portion of exon 2 is also shown with the 2 possible start codons underlined. The 1*Y* region found in the yellow quails is aligned to the homologous region of chicken chromosome 20 and to the 5' non-coding exon of the mouse (Mm) *Raly* gene (accession no. NM_023130). The left hand panels show expression, detected by RT-PCR, of each of the non-coding exons and the 1*Y* transcript, in yellow ($Y/y^+$) and wild-type ($y^+/y^+$) dorsal and ventral skin samples, amplified using forward primers as indicated on the right hand panel and a reverse primer within the coding region of *ASIP* (see text for details).

**Yellow *(Y)* and *ASIP* in Japanese quail**

Sequencing of almost the entire coding region of *ASIP* in the two families segregating for the yellow mutation revealed two variable nucleotide positions (30 and 99) both of which were synonymous. These positions appeared to be in complete linkage disequilibrium with all alleles either being C30T99 or T30C99. In addition the yellow phenotype was associated with the T30C99 allele ($p<0.001$, Fisher's exact test), with

all yellow individuals having at least one of these alleles (table 5.4). This would be consistent with the *Y* allele (which is present in a single copy in the yellow individuals) being linked to a T30C99 *ASIP* allele, while the $y^+$ alleles can be T30C99 or C30T99 in composition. This suggests that, although the yellow mutation does not appear to be due to coding sequence variation at *ASIP,* it is due to a mutation closely linked to this region.

| Nucleotide position | 30 | 99 |
|---|---|---|
| $Y/y^+$ family 1 (n=6) | T/C | C/T |
| $y^+/y^+$ family 1 (n=6) | C | T |
| $Y/y^+$ family 2 (n=6) | T | C |
| $y^+/y^+$ family 2 (n=6) | T/C | C/T |

Table 5.4 Association of the two variable nucleotide positions in the ASIP coding sequence with *Y* locus genotype in 2 families segregating for the yellow phenotype. Both nucleotide positions were synonymous. No variation was found between individuals within families for each of the *Y* genotypes.

Amplification of the non-coding exons of *ASIP* described above revealed the expected pattern with exons 1a and 1c but not 1b amplifying from the dorsal skin samples from both the yellow and wild-type individuals. However sequencing of the amplicons containing exons 1a and 1c from the yellow individuals from family 1 revealed that these transcripts contained only the C30T99 allele, which had been found to be associated with the $y^+$ allele. Similar results were not found for wild-type individuals containing both C30T99 and T30C99 alleles. Therefore it appears that the yellow mutation causes a change in the 5' noncoding regions of *ASIP* and that *Y* alleles do not contain the normally-occurring 5' non-coding exons. This would be consistent with the yellow mutation being due to a change in *ASIP* gene regulation. 5' RACE revealed a novel 5' non-coding region associated with the *Y* allele, which

matched a region of the chicken chromosome 20 over 200Kb upstream of the start of chicken *ASIP* (figure 5.4). This is upstream of the chicken R*aly* gene and this region shows reasonable homology to the 5' non-coding regions of the mouse *Raly* gene. Therefore, it seems possible that the yellow mutation is a large deletion removing the *Raly* gene, placing *ASIP* under the control of the *raly* promoter and causing a *Raly* non-coding exon to be found at the 5' end of the *ASIP* gene.

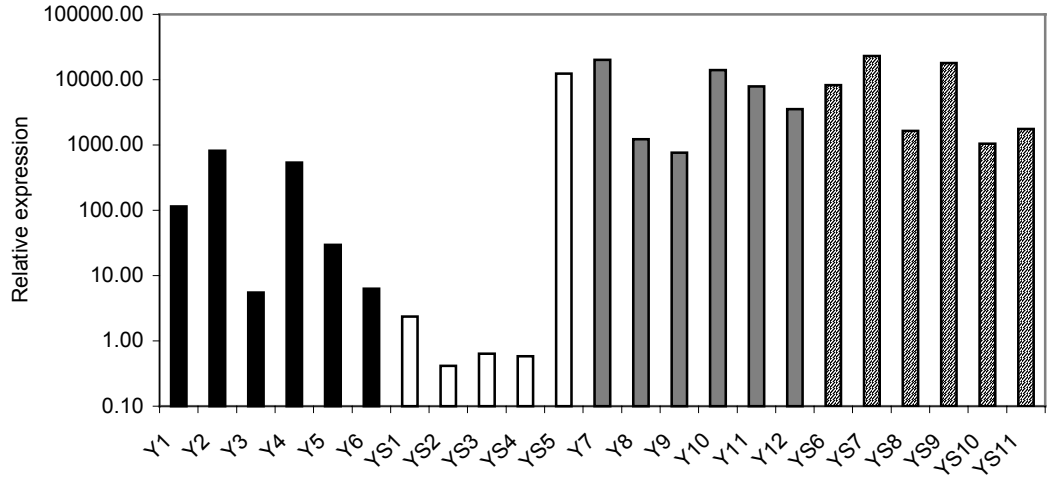



Figure 5.5 *ASIP* expression in plucked and unplucked, yellow and wild-type quails. Black bars = unplucked yellow; white bars = unplucked wild-type; grey bars = plucked yellow; hatched bars = plucked wild-type. Relative expression in shown on a log scale to allow all points to be visualised on the same scale.

A comparison of expression of transcripts containing the coding region of *ASIP* revealed no significant difference between the yellow and wild-type individuals in either the plucked or unplucked families (t-test, p=0.84 and p=0.42 respectively) (figure 5.5). However in the unplucked family the higher expression among the wild-type quails was due to a single outlying wild-type individual (YS5), which had much higher *ASIP* expression than any of the other individuals. If this individual was removed from the analysis the yellow individuals showed on average 251 times higher expression of *ASIP*, but this was still not significant (p=0.13, t-test) due to high variation among the yellow individuals. All other unplucked individuals seem to show

generally lower expression, which is consistent with plucking causing upregulation of expression of *ASIP*.

**Sex differences in gene expression in Common Pheasant Skin**

*TYRP1* showed a sexually dimorphic expression pattern, with significantly higher expression in female than male skin samples (p=0.014, t-test) (table 5.5). *DCT* also had on average higher expression in females than males, although this difference was not significant (p=0.11, t-test). In contrast *MITF* expression was marginally non-significantly higher in males than females (p=0.056, t-test). Average expression of *ASIP* was also higher in males than females, although again this difference was not significant (p=0.36, t-test). Normalising to *β-actin* and *GAPDH* produced very similar results in all cases (table 5.5).

| | Expression (m:f) normalised to | | | |
|---|---|---|---|---|
| Gene | *GAPDH* | p value | *β-actin* | p value |
| *TYRP1* | 0.22 (7/7) | 0.01 | 0.19 (7/7) | 0.01 |
| *MITF* | 3.06 (7/7) | 0.10 | 3.33 (7/7) | 0.05 |
| *DCT* | 0.58 (8/8) | 0.11 | nd | |
| *ASIP* | 4.69 (7/7) | 0.36 | 5.37 (7/7) | 0.34 |

Table 5.5 Male: female ratios for expression of pigmentation genes normalised to expression of *GAPDH* or *β-actin*. (n/n) = n males/ n females, nd = not done. p values indicate significance in 2-tailed t-tests assuming unequal variance.

## Discussion

The extended brown and roux phenotypes of the Japanese quail appear to be caused by point substitutions at the *MC1R* and *TYRP1* loci respectively. The evidence is particularly convincing in the case of *MC1R* given that the same amino acid substitution (Glu92Lys) has been found to produce a dark phenotype in mice, chickens and bananaquits (Robbins et al. 1993; Takeuchi et al. 1998; Theron et al. 2001) and to produce a constitutively active receptor in vitro (Ling et al. 2003).

The phenotypic effect of the Glu92Lys mutation is relatively mild in quail compared to the other cases. For example, mice and bananaquits with a single Glu92Lys allele exhibit eumelanin deposition throughout most hairs or feathers. In chickens, the phenotypic effect of Glu92Lys is abrogated by a second mutation in *MC1R*, His215Pro, as occurs in the buttercup *($e^b$)* allele. However, there are no good candidates for such intragenic epistatic effects in *MC1R* in quails. Comparing the strongly melanic chicken *E* allele with Glu92Lys to the quail *E* allele reveals two fixed differences (Ala24Thr, Leu177Phe) (table 5.6), which are both conservative and unlikely to affect function. Similarly I found no fixed differences between the quail with a darker *E* phenotype and those with the paler *E* phenotype. The unsequenced parts of *MC1R* in this study do not contain residues of known functional significance. Therefore, the mild phenotypic effect of Glu92Lys mutation in most of the quail in this study seems most likely attributable to epistatic effects at other loci.

| Species | Allele | 24 | 33 | 37 | 58 | 71 | **92** | 110 | 126 | 133 | 143 | 177 | 213 | 215 | 244 | Accession no. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Japanese | $e^+$ | T | C | D | V/I | M | **E** | I | V | L | T | F | C | H | L | DQ395091 |
| quail | E | T | C | D | V/I | M | **K** | I | V | L | T | F | C | H | L | DQ395089/90 |
| Red | $e^+$ | A | C | D | V | M | E | I | V | L | L | F | C | H | L | AY220303[1] |
| junglefowl | E | A | C | D | V | M/T | K | T/I | V/I | L | T | L | R | H | L | AY220304[1,2] |
| | $E^{R\text{-}Fayoumi}$ | A | C | D | V | M | E | I | V | Q | T | L | C | H | L | [3] |
| | $e^{bc}$ | A | C | D | V | T | K | I | V | L | T | L | C | P | L | AY220305[1] |
| | $e^y$ | A | W | G | V | M | E | I | V | L | A | L | R | H | P | [2] |

Table 5.6 *MC1R* variation in Japanese quail and domestic chicken. Bold letters indicate amino acid site associated with extended brown in the Japanese quail. [1]sequences reported in Kerje et al (2003), [2]sequences reported in Takeuchi et al (1996), [3]sequences reported in Takeuchi et al (1998).

In chapter 3 I reported Glu92Lys mutations in two further Galliform taxa, the black curassow (*Crax alector*) and the peacock pheasants (*Polyplectron*). There have therefore been at least four independent occurrences of the Glu92Lys mutation in the Galliformes, each associated with the same G272A transition. This suggests high mutability at this nucleotide site in *MC1R* and raises the possibility that this position could act as an evolutionary switch to increase eumelanin deposition.

The evidence for the roux phenotype being caused by the *TYRP1* Phe282Ser mutation is less clear-cut. *TYRP1* coding sequence variants with an effect on phenotype have been found in mice, dogs, cats and cattle (Zdarsky et al. 1990; Schmutz et al. 2002; Berryere et al. 2003; Schmidt-Kuntzel et al. 2005) and none of these have involved an amino acid at this position (table 5.7). However, an alignment of *TYRP1* protein sequences from a wide range of taxa including several mammal species and the zebra fish revealed that this amino acid position is conserved as a phenylalanine in all these taxa suggesting it is important for enzyme function. There is still a possibility that the causative mutation could be at a site closely linked to this one for example in the regulatory regions of *TYRP1*. The lack of a significant

difference in *TYRP1* expression between the roux and wild-type individuals makes this unlikely, although a slight non-significant reduction in *TYRP1* expression is present in the roux quails. However, a similar reduction in expression is seen in *MITF,* suggesting that this is due to a regulatory event upstream of *TYRP1*, perhaps due to some feedback mechanism responding to the *TYRP1* mutation. Regulatory mutations of *TYRP1* have been described in mice, which lead to *TYRP1* mRNA levels of about 1% of wild-type levels (Jackson et al. 1990). Heterozygotes for this mutation, which produced about half normal levels, exhibit a wild-type phenotype. This suggests that the small reduction in *TYRP1* expression seen in the roux quails is unlikely to be the cause of the change in phenotype.

The phenotypic effect of the *TYRP1* mutation in the roux quails seems more pronounced than the effect of most of the previously described mutations at this locus in mammals. The brown mouse, chocolate cat and dun Dexter cattle phenotypes and the brown coat colour of dogs are all darker than roux phenotype in quails. The cinnamon ($b^l$) allele in cats produces a similarly light phenotype but is due to a premature stop codon at position 100 of *TYRP1* (Schmidt-Kuntzel et al. 2005). This would be expected to have a more extreme effect on phenotype than the single amino acid substitution found in the roux quails. This raises the possibility that the Phe282 residue is particularly important for enzyme function, with change at this position having a larger effect than the other described point substitutions. This residue does not lie in the copper binding domains at positions 215-232 and 397-409 (April et al. 1998b), which are known to be essential for enzyme function, but does lie within a cysteine-rich region thought to be important for enzyme structure. An alternative possibility is that the paler phenotype in quails is due to epistatic effects. In this regard

it would be interesting to characterise the quail *brown* allele at this locus to see how it compares to the brown alleles in the other taxa.

| Species | Allele | 3 | 41 | 100 | 110 | 166 | 282 | 331 | 345 | 384 | 421 | 434 | Accession no. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Quail | $BR^+$ | L | C | R | C | R | F | E | P | L | A | H | |
| | $br^r$ | L | C | R | C | R | **S** | E | P | L | A | H | |
| Chicken | $BR^+$ | L | C | R | C | R | F | E | P | L | A | H | AF003631[2] |
| Mouse | $B^+$ | S | C | Q | C | L | F | Q | P | L | A | H | NM_031202 |
| | *b* | S | C | Q | **Y** | L | F | Q | P | L | A | H | [3] |
| Dog | $B^+$ | A | C | H | C | S | F | Q | P | L | A | H | AY052751[4] |
| | $b$ [1] | A | **S** | H | C | S | F | **X** | **Δ** | L | A | H | [4] |
| Cat | $B^+$ | A | C | R | C | S | F | Q | P | L | A | H | AY956310[5] |
| | *b* | **G** | C | R | C | S | F | Q | P | L | A+**16/17** | H | AY965744/5[5] |
| | $b^l$ | A | C | **X** | - | - | - | - | - | - | - | - | AY965746[5] |
| Cow | $B^+$ | S | C | H | C | S | F | Q | P | L | A | H | NM_174480[6] |
| | *b* | S | C | H | C | S | F | Q | P | L | A | **Y** | [6] |
| Human | $B^+$ | A | C | H | C | S | F | Q | P | L | A | H | BC052608 |
| | $b^{OCA3}$ [1] | A | C | H | C | **X** | F | Q | P | **X** | - | - | [7] |
| Horse | $B^+$ | A | C | H | C | S | F | Q | P | L | A | H | BK000021[8] |
| Pig | $B^+$ | A | C | H | C | S | F | Q | P | L | A | H | NM_001025226 |
| Zebrafish | $B^+$ | - | C | Q | C | F | F | Q | P | L | A | H | BC076406 |

Table 5.7 *TYRP1* sites associated with diluted pigmentation in mammals and birds. Bold letters indicate the variant amino acids associated with the mutant allele. - = amino acid absent, X = stop codon, Δ = deletion, +n = insertion of n length. [1]At these alleles, any one of the highlighted variant positions in homozygous or two in heterozygous state are sufficient to produce a pale phenotype. [2]Reported in (April et al. 1998b), [3]reported in (Zdarsky et al. 1990), [4]reported in (Schmutz et al. 2002), [5]reported in (Schmidt-Kuntzel et al. 2005), [6]reported in (Berryere et al. 2003), [7] reported in (Boissy et al. 1996; Manga et al. 1997), [8]reported in (Rieder et al. 2001)

In contrast to the previous two mutations the yellow mutation does not seem to be caused by a change in coding sequence. However the significant association between the *Y* allele and two synonymous single nucleotide polymorphisms (SNPs) within the coding region of *ASIP* strongly suggests that *Y* is an allele of this locus. The situation seems very similar to that produced by the mouse lethal yellow mutation. The quail *Y* allele appears to be under the control of a different promoter from the

normal *ASIP* alleles, which lies about 200kbp upstream and is upstream of the *Raly* gene. It seems likely that as in mouse $A^y$ this is the *Raly* promoter and that *ASIP* is controlled via this because of a deletion removing the *Raly* gene. Although I was not able to confirm a deletion, this is consistent with the homozygous lethality of the *Y* allele, which in mice is thought to be due to the complete loss of the *Raly* gene. If this hypothesis were correct the expectation would be for higher *ASIP* expression in the yellow quails. However I failed to find a significant difference in expression between $Y/y^+$ and $y^+/y^+$ quails. There seem to be two possible explanations for this incongruity. The first is that the novel promoter of the *Y* allele does not increase the production of ASIP above normal levels but instead produces an mRNA molecule that is more efficiently transcribed or is translated into a more potent protein. This type of effect could have been mediated via an alternative start codon as is found at *ASIP*. However it seems that use of the alternative start codon would not produce a more effective signal peptide and so would be unlikely to have an effect on ASIP activity.

The second, and perhaps more likely, explanation is that the *Y* allele does cause increased expression of *ASIP* but that this is masked by the high variation in the background levels of *ASIP* expression in the skin samples in this study. In mice, as well as being involved in producing the pale ventrum, *ASIP* is also involved in producing banded (agouti) hairs on the dorsum (Vrieling et al. 1994). If *ASIP* also has this role in birds, i.e. producing within-feather banding or patterning, then expression would be expected to vary across the feather growth cycle and be high when pale feather bands are being produced. This would explain why plucking caused increased *ASIP* expression; this would re-set the feather growth cycle and the tips of quail feathers tend to be pale. It would also explain why there was less variation in *ASIP* expression in the plucked compared to unplucked samples, as the plucked samples

would all be at the same stage of feather growth, whereas the unplucked samples could have been at different stages. Similarly a greater difference was seen between the yellow and wild-type individuals in the unplucked samples than plucked samples.

In order to overcome the problem of variable *ASIP* expression it may be necessary to take skin samples from embryos before feather growth begins when wild-type *ASIP* mRNA levels would be expected to be low. It will also be interesting to investigate expression of *ASIP* in tissues other than skin to see if the yellow quails, like the yellow mice, show ubiquitous expression. Yellow quails do not seem to show evidence of overeating or obesity, as is found in yellow mice, although this has not yet been checked in detail. The melanocortin system of the central nervous system does appear to be active in Japanese quail and involved in feeding and energy homeostasis (Boswell et al. 2002; Boswell and Takeuchi 2005). Therefore the lack of overeating could indicate that *ASIP* is not expressed in the brain of the yellow quails or that avian *ASIP* has a low or no affinity for the melanocortin receptors in the brain. The latter seems valid given that avian *ASIP* and *AGRP* show less homology than those of mammals (Klovins and Schiöth 2005).

The finding of an expressed *ASIP* gene in birds that is involved in dorsoventral pigmentation patterning is interesting as it suggests a conserved basis for this type of pattern between birds and mammals. This backs up evidence suggesting that *ASIP* diverged from *AGRP* early in vertebrate evolution (Klovins and Schiöth 2005) and that *ASIP* may be involved in regulating dorsoventral pigmentation patterning in fish (Cerda-Reverter et al. 2005). Fish differ from mammals and birds in that they have several different types of pigment cells (chromatophores); only one of these contains melanin (the melanophores) and only eumelanin not pheomelanin (Ito and Wakamatsu 2003; Kelsh 2004). In fish *ASIP* is expressed only in the ventral and not

the dorsal skin and seems to be involved in directing chromatophore differentiation, causing production of iridophores (structural pigment cells) and inhibiting production of melanophores. Therefore it seems that the genetic pathways for bringing about pale ventral and dark dorsal pigmentation are ancient and conserved within the vertebrates even though the cellular and biochemical basis of these patterns has changed, perhaps independently, in the birds and mammals. All three of the quail pigmentation variants studied here show striking similarity at the molecular level to mutations found in mammals. This illustrates the similarity between the pigmentation systems of birds and mammals and also the validity of a candidate gene approach in investigating avian pigmentation genetics.

Investigation of sexual differences in candidate gene expression in common pheasant skin revealed expression patterns consistent with the observed differences in plumage colour. Male pheasants showed higher *MITF* expression, consistent with a higher overall level of melanin synthesis. However they also showed significantly lower levels of *TYRP1* expression. This is consistent with a reduced level of eumelanin synthesis and a high level of pheomelanin synthesis that would be necessary to produce the strong red colour of male pheasant body feathers. Also consistent with this pattern, *DCT*, which is like *TYRP1* in only being involved in eumelanin synthesis, was lower in males and *ASIP* was higher in males. However, neither of these differences was significant suggesting that these loci are not as important for generating sexual dichromatism, at least in the sampled skin region.

It is interesting that *TYRP1* is the only locus to show a significant sex difference in expression and is also the only sex-linked locus among those studied. Unlike mammals, birds do not seem to show ubiquitous dosage compensation, although the majority of genes do seem to be compensated (McQueen et al. 2001).

The null expectation would therefore be that males and females would either show the same levels of *TYRP1* expression or that males would show levels twice as high as females. That the expression difference goes in the opposite to expected direction strongly suggests that *TYRP1* expression is regulated directly or indirectly by sex hormone receptors in the skin. In pheasants sexual dichromatism seems to be most likely to be oestrogen–dependant (Kimball 2006), suggesting that oestrogen may increase *TYRP1* transcription in female common pheasants.

Sex chromosomes have been reported to show a disproportionate effect on sexually selected or sex-limited traits across a range of animals with XY or ZW sex determination systems (Reinhold 1998; Sætre et al. 2003). It has been proposed that this may be due to a faster evolving Z chromosome, due to male-biased mutation, which would promote the evolution of rapidly evolving male displays or traits involved in population divergence on this chromosome (Kirkpatrick and Hall 2004). Alternatively, Z-linked traits that are beneficial to males but detrimental to females could be favoured in the early stages of the evolution of a sexually selected trait because sons would be more likely to inherit them than daughters (Albert and Otto 2005). In a study of *Ficedula* flycatchers, Sætre *et al* (2003) found that 3 sex-limited male plumage characteristics were determined by Z chromosome not autosomal genotype. My finding of low relative expression in males of a Z-linked gene involved in pigmentation is further evidence that Z-linked loci are involved in the production sex-specific plumage traits and suggests a possible mechanism for this in birds. However the expression difference is in the opposite to expected direction for some of the models given that if it arose due to a mutation on the Z chromosome it would presumably have been recessive and therefore been exposed to selection at lower frequency in males than in females. Although this result is intriguing, sampling of

many more loci is necessary to establish the relative importance of sex-linked as compared to autosomal loci.

Overall this study illustrates the utility of a candidate gene approach in avian pigmentation genetics, from the basic level of identifying the genetic basis of specific pigmentation mutations in domestic birds to more complex questions such as the genetic basis of sexual dichromatism in semi-wild birds.

# Chapter 6

# Concluding thoughts

In this final chapter I try to bring together my findings from the previous chapters and highlight some areas of particular interest and possible avenues for further research. Results from my work on Japanese quail pigmentation variants have implications for my earlier comparative study of pigmentation gene evolution. In particular the finding that *TYRP1* is functional in birds and that mutations at this locus can have an effect on phenotype, back up the use of this locus in the comparative study. However, the finding of an active *ASIP* gene in birds, suggests that *AGRP* is unlikely to function in pigmentation in birds and so in hindsight this locus was less relevant and hence it was used only as a control. It also raises the possibility of investigating *ASIP* evolution across the Galliformes as this gene may share many of the properties of *MC1R* that make it amenable to directional selection acting on colour. The role of *ASIP* in producing a pale-bellied phenotype would also be interesting to investigate further across more avian species, given the repeated loss or modification of this widespread colour scheme in birds.

## Plumage colour evolution in the Galliformes

Studies of the Galliformes have provided some of the classical demonstrations of plumage colour being used for female choice (Petrie et al. 1991; Zuk et al. 1992) or male-male competition (Mateos and Carranza 1997). An interesting feature of plumage colour evolution in the galliform birds is its possible role in species divergence. In at least one gallinaceous species, the sage grouse (*Centrocercus urophasianus*), an isolated population was found to show rapid change in male secondary sexual characters, including plumage colour (Young et al. 1994). I found evidence for a punctuational mode of evolution of sexual dichromatism, suggesting that changes in plumage colour may be important in the speciation process. In addition the pattern of ongoing change at *MC1R* on lineages with high dichromatism is suggestive of the type of ongoing sexual selection that may be expected to lead to radiations of male ornaments that may contribute to speciation (Mead and Arnold 2004). Many, even apparently distantly related galliform species, can hybridise in captivity or even at a very low rate in wild sympatric species (Danforth 1950; Madge and McGowan 2002; Minvielle et al. 2002). Therefore the male plumage traits of these birds may play an important role in pre-mating species isolation.

The comparative simplicity of the pigmentation of galliform feathers in terms of their lack of carotenoids makes them a useful study system. It also means that the genetic control of melanin synthesis and deposition is likely to be particularly important. My finding of a higher rate of evolution at *MC1R* associated with higher levels of sexual dichromatism backs this up, suggesting that control of melanin synthesis has been an important component of the evolution of ornamental plumage colours of these birds. This is perhaps surprising given that the characterised *MC1R* mutations have primarily caused switches to pure black or highly melanic colouration

(Takeuchi et al. 1998; Theron et al. 2001; Eizirik et al. 2003; Nachman et al. 2003; Mundy et al. 2004), while male ornamental plumage colour in the Galliformes is highly diverse with structural blues and greens and red colours often exhibited. However *MC1R* substitutions can also cause reductions in eumelanism, often in association with increases in pheomelanism, a prominent example being the variants associated with human red hair colour (Valverde et al. 1995; Schiöth et al. 1999). In addition a possible association between an *MC1R* variant and change in a structural colour has been found (Doucet et al. 2004), albeit only through a blocking of the colour by increased melanin deposition. This does raise the possibility, however, that mutations of smaller effect at *MC1R* could cause changes structural colours. It is not my suggestion that all plumage colour variation seen in the Galliformes can be explained by *MC1R* variation, as this is almost certainly controlled by a large number of loci (Danforth 1950). An interesting question is if other loci have also repeatedly been used in the evolution of male ornamental plumage colour in the Galliformes. One possibility seems to be a locus involved more directly in the production of structural colours.

## Pigmentation gene evolution and implications for sexual selection

One of the implications of my findings is that loci that have been under sexual selection in the past will not necessarily be those that currently show evidence of sexual selection in present populations. Despite evidence for sexual selection acting on *MC1R* on certain lineages, a very low level of amino acid variation was found in a population of black grouse - a species on one of these lineages. This suggests very little potential for ongoing sexual selection at this locus in present populations, which is supported by evidence suggesting that plumage is not used for female choice or male competition in this species (Alatalo et al. 1991; Rintamaki et al. 1997). Quantitative genetic studies of sexual selection in avian populations have focused on the importance of having heritable genetic variation in traits under sexual selection (Norris 1993; Pomiankowski and Møller 1995; Griffith et al. 1999a; Qvarnström 1999a; Török et al. 2003; Qvarnström et al. 2006). While this may be necessary for ongoing sexual selection at a particular locus, my results suggest that its absence does not necessarily rule out the possibility of a historical effect of sexual selection.

The importance of sex-linkage in loci controlling sexually selected traits is an area of current debate (Bartosch-Härlid et al. 2003; Sætre et al. 2003; Kirkpatrick and Hall 2004; Albert and Otto 2005; Johns et al. 2005; Mank et al. 2006). I only investigated one sex-linked locus (*TYRP1*) and therefore general inferences about the importance of sex-linked loci are only tentative. However my finding of a mutation at this locus with an effect on pigmentation phenotype, suggests that *TYRP1* is a candidate for a sex-linked locus with the potential to influence male ornamental plumage colour. This is supported by its differential expression in male and female common pheasants. I did not find evidence for rapid change at this locus associated with sexual selection, as may be expected if it was evolving in response to sexual

selection. However, there appeared to be a higher level of purifying selection acting at this locus in lineages with higher sexual selection. This suggests that *TYRP1* may be involved in the production of male ornaments and so subject to higher constraint due to female choice. These findings raise the possibility that regulatory regions of *TYRP1* could be evolving in response to sexual selection to produce male ornamental traits.

Another interesting finding of possible importance is the repeated use of the same locus (*MC1R*) in sexually dimorphic evolution on distantly related lineages. Two possible sexual selection scenarios could explain this occurrence. The first being condition dependence: if a particular locus was involved in the production of a trait with potential for condition dependence it may be repeatedly used on several lineages. The potential of melanin based plumage colour to be condition-dependent has been argued and there does seem to be potential for high eumelanin or pheomelanin to be costly (Veiga 1993; McGraw 2003; Parker et al. 2003; Takeuchi et al. 2004). The second scenario is sensory bias: if the same locus is repeatedly selected because it produces a trait with the potential to stimulate the female sensory system (Fuller et al. 2005). This is perhaps less likely given that, although there may be convergence in one of the loci involved, there does not appear to be a high degree of convergence in the types of plumage colour traits produced on different lineages.

Alternatively the repeated use of some loci in phenotypic evolution on different lineages may not be a unique feature of sexual selection but a more general feature of the selective process as a whole. This is supported by previous studies showing *MC1R* variation associated with colour variation, apparently evolving in response to natural selection (Theron et al. 2001; Hoekstra et al. 2004; Rosenblum et al. 2004). This may be because some key loci, such as *MC1R*, show high tissue and functional specificity and therefore have few if any pleiotropic effects. This means

that they are relatively free to evolve independently of other traits (Mundy 2005; Brakefield 2006; Griswold 2006).

## The evolutionary genetics of avian pigmentation

An interesting question in evolutionary biology is the production of convergent traits and whether these are convergent at the genetic level or due to different loci with similar effects (Price and Pavelka 1996; Omland and Lanyon 2000; Brakefield). I found that the glutamate-92-lysine substitution at *MC1R*, which has been found to cause melanism in chickens, bananaquits and mice (Robbins et al. 1993; Takeuchi et al. 1998; Theron et al. 2001), is also responsible for producing a dark phenotype in Japanese quails and occurred independently two further times in the galliform phylogeny. This raises the possibility that convergent phenotypes could be caused by particularly mutable genetic switches within certain loci. However it is worth noting that the phenotypes produced by this mutation differ between taxa, highlighting the importance of the genetic background on which they occur.

One of the large remaining questions in the genetics of avian pigmentation concerns the production of regional and within-feather patterning. It appears that *ASIP* plays a role in dorso-ventral patterning and the possibility that it may have a wider role in both additional types of body region patterning and within-feather patterning is intriguing. Reaction-diffusion models have been used successfully to describe the production of both body region and with-feather patterns (Price and Pavelka 1996; Prum and Williamson 2002). These models involve the presence of an activator molecule, which shows auto-activation and activates an inhibitor molecule, which inhibits the activation molecule (figure 6.1). It seems unlikely that *ASIP* and *MSH* are themselves the activators and inhibitors, as they have not been described to have the necessary activation and inhibition properties. Rather it seems more likely that the activator and inhibitor act upstream to determine the transcription of *ASIP* and *MSH*. A particularly puzzling question is how the temporal expression of the molecular

components is regulated differently between feather follicles at different locations on the body in order to produce body-level differences in within-feather patterns.

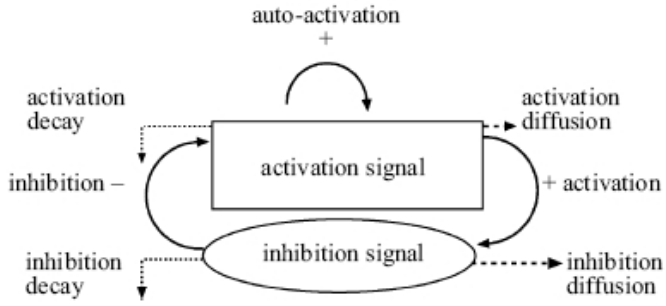



Figure 6.1 A reaction-diffusion system, from Prum (2002). The activating signal reinforces its own production and the production of an inhibitory signal, which inhibits the production the activating signal. Both signals diffuse and decay over time.

The peacock's tail is the most prominent of example of sexual selection in the Galliformes, with the number of a male's eye-spots determining his mating success (Petrie et al. 1991). The evolution and development of these eye-spots is intriguing. This pattern was found to be one of the hardest within-feather patterns to simulate using a reaction-diffusion model (Prum and Williamson 2002). This is particularly interesting given that similar patterns of large medially placed feather spots are found in two other Phasianidae genera, the peacock pheasants and the argus pheasant (figure 6.2a-c). My phylogenetic reconstructions suggest that these taxa are not monophyletic, suggesting that these patterns either evolved independently on these lineages or are ancestral to a large portion of the Phasianidae. Complex within-feather patterns appear to be fairly common within the Galliformes, with other examples including the complex spotting of guineafowl feathers (figure 6.2d).

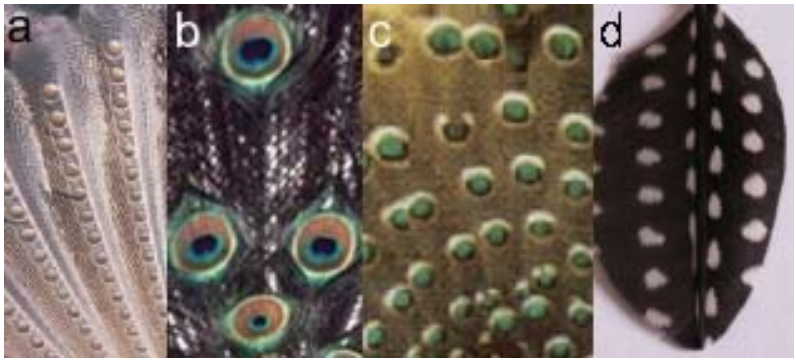



Figure 6.2 Spotted feather patterns within the Galliformes. **a**, great argus, *Argusianus argus*, wing feathers in display. **b**, blue peacock, *Pavo cristatus*, tail feathers in display. **c**, grey peacock pheasant, *Polyplectron bicalcaratum*, wing feathers in display. **d**, helmeted guineafowl, *Numida meleagris*, feather. Photographs (a-c) courtesy of J. Corder.

## To conclude

The characterisation at the molecular level of melanin synthesis and deposition within the birds is in its early stages. I have contributed to this by confirming the presumed functions of *TYRP1* and *ASIP* in avian pigmentation. The genetics of melanin synthesis appears to be very similar between both birds and mammals, which should help improve our understanding and the identification of candidate genes for further evolutionary studies. However we still know almost nothing about the genetics of structural, carotenoid or other types of non-melanic pigmentary feather colours.

The investigation of sexual selection at the molecular level is a very new field (Civetta 2003) and the precise patterns of DNA sequence evolution expected under sexual selection have not yet been established, especially for secondary sexual traits such as colour. Quantitative genetic models assume a large number of loci determining the phenotype of a trait summarised by a parameter describing the genetic variance at these loci (Mead and Arnold 2004). In this regard a new class of model may be necessary in order to test sexual selection hypotheses using molecular genetic data. In particular very few predictions have been made regarding the number or type of loci that should be involved in the production of ornamental traits or how conserved these should be across different taxa.

I have identified what may be a signature of sexual selection driving change at *MC1R* and a possible effect of sexual selection causing constraint at two further pigmentation loci. The interpretation of these findings in terms of sexual selection theory is currently far from obvious but will become clearer as more theoretical and empirical work at the molecular level is performed. It will be particularly interesting to find out if similar results can be found at other loci, including those involved in the production of other types of secondary sexual traits.

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# Appendix 1 Table of abbreviations

| | |
|---|---|
| AGRP | Agouti related protein |
| ASIP | Agouti signalling peptide |
| BMP2 | Bone morphogenic peptide 2 |
| BMP4 | Bone morphogenic peptide 4 |
| cAMP | Cyclic adenosine monophosphate |
| cDNA | Complementary DNA |
| CYTB | Cytochrome B |
| DCT | DOPAchrome tautomerase |
| df | Degrees of freedom |
| dN | Rate of nonsynonymous substitution |
| DNA | Deoxyribonucleic acid |
| DOPA | Dihydroxyphenylalanine |
| dS | Rate of synonymous substitution |
| Edn3 | Endothelin-3 |
| EDNRB | Endothelin receptor-B |
| EDNRB2 | Endothelin receptor-B subtype-2 |
| EST | Expressed sequence tag |
| GAPDH | Glyseraldehyde-3-phosphate dehydrogenase |
| LRSt | Likelihood ratio statistic |
| LRT | Likelihood ratio test |
| MC1R | Melanocortin 1-receptor |
| MCMCMC | Metropolis-coupled Markov chain Monte Carlo |
| MITF | Microphthalmia-associated transcription factor |
| MK | McDonald-Kreitman (test) |
| Mlph | Melanophilin |
| MSH | Melanocyte stimulating hormone |
| OCA | Oculocutaneous albinism |
| PCR | Polymerase chain-reaction |
| PMEL17 | Melanosomal protein 17 |
| POMC | Pro-opiomelanocortin |
| RIN | RNA integrity number |
| RNA | Ribonucleic acid |
| RT-PCR | Reverse transcriptase PCR |
| SHH | Sonic hedgehog |
| SNP | Single nucleotide polymorphism |
| TYRP1 | Tyrosinase related protein-1 |
| UV | Ultra violet |
| WNT3 | Wingless int3 |
| ω | dN/dS |

# Appendix 2 Samples used in the phylogeny and comparative studies

| Species | | Source | No. of samples (individuals) | *MC1R* | *TYR* | *TYRP1* | *DCT* | *AGRP exons* | *AGRP introns* | *CYTB* |
|---|---|---|---|---|---|---|---|---|---|---|
| Vulturine guineafowl | *Acryllium vulturinum* | NMS | 2 | DQ395094 | EF571143 | EF571109 | EF571072 | EF571219 | EF571178 | *AF536742* |
| Wattled Brush-turkey | *Aegypodius arfakianus* | NMS | 1 | | EF571127 | EF571093 | EF571074 | | | |
| Congo peafowl | *Afropavo congensis* | NMS | 3 | EF571041 | EF571146 | EF571112 | EF571076 | EF571221 | EF571181 | *AF013760* |
| Chukar | *Alectoris chukar* | NMS | 1 | EF571038 | EF571145 | EF571111 | EF571075 | EF571220 | EF571180 | *L08378* |
| Red-legged partridge | *Alectoris rufa* | NM | 1 | EF571029 | EF571144 | EF571110 | EF571073 | EF571223 | EF571179 | *Z48775* |
| Great Argus | *Argusianus argus* | NHM | 1 | EF571018 | EF571148 | EF571102 | EF571069 | EF571222 | EF571169 | *AF013761* |
| Cheer Pheasant | *Catreus wallichi* | NHM | 1 | | | | | | | |
| Cheer Pheasant | *Catreus wallichi* | WPA(JC) | 1 | EF571015 | EF571138 | EF571104 | EF571067 | EF571213 | EF571172 | *AF028792* |
| Common Quail | *Coturnix coturnix* | INRA(FM) | 2 | DQ395093 | EF571140 | EF571106 | EF571077 | EF571216 | EF571175 | *L08377* |
| Japanese Quail | *Coturnix japonica* | INRA(FM) | 2 | DQ395091 | *AB024278* | *AB005228* | *AB081466* | EF571215 | EF571174 | |
| Black Curassow | *Crax alector* | NMS | 1 | EF571030 | EF571142 | EF571108 | EF571071 | EF571218 | EF571177 | *AY141921*, EF571188 |
| White Eared-pheasant | *Crossoptilon crossoptilon* | NMS | 1 | EF571040 | EF571141 | EF571107 | EF571070 | EF571217 | EF571176 | *AF028794* |
| Brown Eared-pheasant | *Crossoptilon mantchuricum* | NMS | 1 | EF571031 | EF571139 | EF571105 | EF571068 | EF571214 | EF571173 | *AF534553* |
| Grey Francolin | *Francolinus pondicerianus* | NHM | 1 | EF571023 | EF571136 | EF571113 | EF571066 | EF571211 | EF571211 | *U90648* |
| Sonnerat's Jungle Fowl | *Gallus sonneratii* | NMS | 1 | DQ395092 | EF571135 | EF571101 | EF571065 | EF571210 | EF571170 | EF571186 |
| Red Grouse | *Lagopus lagopus scoticus* | GCT(DB) | 3 | EF571024 | EF571131 | EF571097 | EF571060 | EF571206 | EF571166 | EF571187 |
| Himilayan Monal | *Lophophorus impejanus* | NMS | 1 | EF571033 | EF571132 | EF571098 | EF571061 | EF571207 | EF571167 | *AF028796* |
| Siamese fireback | *Lophura diardi* | ZSL | 1 | EF571032 | EF571134 | EF571100 | EF571063 | EF571209 | EF571182 | *AF028797* |
| Edwards's pheasant | *Lophura edwardsi* | NMS | 1 | EF571034 | EF571133 | EF571099 | EF571062 | EF571208 | EF571168 | *AF314638* |
| True silver pheasant | *Lophura nycthemera* | WPA(SW) | 1 | EF571035 | EF571130 | EF571096 | EF571059 | EF571205 | EF571165 | *L08380* |
| Swinhoes pheasant | *Lophura swinhoii* | WPA(SW) | 2 | EF571036 | EF571129 | EF571095 | EF571058 | EF571204 | EF571164 | *AF314644* |
| Turkey | *Meleagris gallopavo* | T | 1 | EF571026 | EF571126 | EF571092 | EF571056 | EF571202 | EF571162 | *L08381* |
| Helmeted Guineafowl | *Numida meleagris* | CS | 2 | EF569209 | EF571125 | EF571091 | EF571055 | EF571201 | EF571161 | *L08383* |
| Indian Peafowl | *Pavo cristatus* | NMS | 1 | EF571039 | EF571124 | EF571090 | EF571054 | EF571200 | EF571160 | *L08379* |
| Green peafowl | *Pavo muticus* | ZSL | 1 | EF571028 | EF571120 | EF571086 | EF571050 | EF571196 | EF571156 | *AF013763* |
| Grey Partridge | *Perdix perdix* | GCT(MB) | 3 | EF571013 | EF571118 | EF571084 | EF571049 | EF571194 | EF571154 | *AF028791* |
| Common pheasant | *Phasianus colchicus* | GCT(MB) | 3 | EF571043 | EF571123 | EF571089 | EF571053 | EF571199 | EF571159 | *AF028798* |
| Palawan Peacock-pheasant | *Polyplectron emphanum* | NHM | 1 | EF571014 | EF571122 | EF571088 | EF571052 | EF571198 | EF571158 | *AF330062* |

| Species | | Source | No. of samples (individuals) | *MC1R* | *TYR* | *TYRP1* | *DCT* | *AGRP exons* | *AGRP introns* | *CYTB* |
|---|---|---|---|---|---|---|---|---|---|---|
| Mountain Peacock-pheasant | *Polyplectron inopinatum* | NHM | 1 | EF571020 | EF571121 | EF571087 | EF571051 | EF571197 | EF571157 | *AF330064* |
| Malayan Peacock-pheasant | *Polyplectron malacense* | NHM | 1 | EF571019 | EF571119 | EF571085 | EF571078 | EF571195 | EF571155 | *AF330065* |
| Crested partridge | *Rollulus rouloul* | ZSL | 1 | EF571027 | EF571117 | EF571083 | EF571048 | EF571193 | EF571153 | EF571185 |
| Orange River Francolin | *Scleroptila levaillantoides* | CS | 3 | EF571017 | EF571137 | EF571103 | EF571066 | EF571212 | EF571171 | EF571184 |
| Reeves's pheasant | *Syrmaticus reevesii* | NHM | 1 | EF571022 | EF571116 | EF571082 | EF571047 | EF571192 | EF571152 | *AF028801* |
| Black grouse | *Tetrao tetrix* | NMS | 1 | EF571037 | EF571128 | EF571094 | EF571057 | EF571203 | EF571163 | EF571183 |
| Capercaillie | *Tetrao urogallus* | NMS | 2 | EF571025 | EF571147 | EF571079 | EF571044 | EF571189 | EF571149 | *AB120132* |
| Satyr Tragopan | *Tragopan satyra* | NHM | 1 | EF571016 | EF571115 | EF571081 | EF571046 | EF571191 | EF571151 | *AF200724* |
| Temminck's tragopan | *Tragopan temminckii* | NHM | 1 | EF571021 | EF571114 | EF571080 | EF571045 | EF571190 | EF571150 | *AF028802* |
| Temminck's tragopan | *Tragopan temminckii* | WPA(SW) | 1 | | | | | | | |

Source codes:
CS Claire Spottiswoode (University of Cambridge)
FM Francis Minvielle (INRA, France)
GCT Game conservency Trust (MB) Malcolm Brockless (DB) David Baines
NHM Natural History Museum, Tring
NM Nick Mundy
NMS National Museum of Scotland
T Tesco Supermaket
WPA World Pheasant Association (SW) Stuart Willson (JC) John Corder
ZSL Zoological Society of London

Genbank accession numbers for each of the loci, where available, are given. Italics indicate sequences downloaded from the databases and not generated in this study. X indicates that sequence data for this locus was generated but has not yet been deposited in genbank.

# Appendix 3 Maximum parsimony phylogeny and dichromatism reconstruction